Structuration of dense casein suspensions by phosphates in the presence of calcium : a study of their organization and rheological properties

Peggy Thomar

UNIVERSITE BRETAGNE LOIRE

Université du Maine

Thèse de Doctorat

Peggy THOMAR

Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université du Maine
sous le sous le sceau de l'Université Bretagne Loire

École doctorale : *3MPL*

Discipline : *Chimie des matériaux*
Spécialité : *Chimie et physico-chimie des polymères*
Unité de recherche : *IMMM, UMR CNRS 6283*

Soutenue le *21 janvier 2016*

Structuration des suspensions denses de caséines par les phosphates en présence de calcium, étude de leurs organisations et de leurs propriétés rhéologiques.

JURY

Rapporteurs :	**Milena CORREDIG,** Professeur, Université de Guelph, Ontario (Canada) **Frédéric GAUCHERON,** Chargé de Recherche, Agrocampus Ouest, Rennes
Examinateurs :	**Maud PANOUILLE,** Maître de Conférences, AgroParisTech, Grignon **Antoine BOUCHOUX,** Chargé de Recherche, INRA, Toulouse **Christophe CHASSENIEUX,** Professeur, Université du Maine, Le Mans
Invité(s) :	**Anne PITKOWSKI,** Chargé de Recherche, Fromageries Bel, Vendôme
Directeur de Thèse :	**Taco NICOLAI,** Directeur de Recherche, Université du Maine, Le Mans

Remerciements.

Je souhaite tout d'abord remercier mon encadrant de thèse le Dr Taco Nicolai pour son aide et sa disponibilité. Je lui exprime toute ma reconnaissance pour les connaissances partagées avec moi et pour les nombreux échanges tout au long des trois années de thèse.

J'adresse mes sincères remerciements à Anne Pitkowski pour sa confiance, son accompagnement et son soutien tout au long de mon parcours. J'adresse également une pensée particulière pour Benoit Goldschmidt et Marie-Hélène Chassagne.

Je remercie l'ensemble des membres du jury, les Pr. Milena Corredig et Christophe Chassenieux, les Dr. Frederic Gaucheron, Maud Panouillé et Antoine Bouchoux d'avoir accepté d'examiner ce travail de thèse.

Je tiens à remercier l'ensemble de l'équipe PCI, tous les permanents et doctorants pour les nombreux moments passés ensemble. Merci pour votre aide, vos conseils et votre assistance. Tout particulièrement, j'exprime toute ma reconnaisance et ma sympathie pour le Pr. Dominique Durand mais aussi pour mes amis docteurs Damien, Anna et Andrii. Je vous remercie pour tous vos conseils, votre amitié, votre humour et tous les moments passés ensembles.

Je pense également à mes amis successeurs, Walailuk et Frederico. Je vous souhaite plein de réussite dans vos travaux et pour vos carrières futures.

Je remercie enfin ma famille qui m'a toujours supportée dans les moments les plus difficiles. Merci d'avoir toujours été présents et de l'être toujours aujourd'hui.

Contents

General introduction

Casein is the most abundant milk protein. Four casein molecules (α_{s1}, α_{s2}, β and κ-casein) are associated into a complex called the casein micelle. The caseins are held together by colloidal calcium phosphate (CCP) that maintains the integrity of the micelle. The casein micelle is relatively stable, but can be disrupted when the physico-chemical environment is modified, which is exploited for producing many dairy products. When CCP is removed by polyphosphates the casein micelle is dissociated into smaller particles called "submicelles". Generally, the submicelles aggregate and create a strong network with crosslinks that are mediated by calcium and phosphates. When casein micelles are acidified at pH 4.6, the CCP is completely dissolved and the proteins precipitate. After elimination of the CCP, the caseins can be neutralized with NaOH to obtain sodium caseinate (NaCas). In aqueous solution, sodium caseinate caseins are also associated into submicelles.

At the start of this PhD research it was already known addition of calcium ions to dilute NaCas solutions leads to an increase of the turbidity caused by larger scale aggregation of casein. However, the behaviour in concentrated solutions and the possibility of gelation of casein induced by calcium had not yet been investigated. The presence of phosphate in addition to calcium changes the interactions that drive aggregation and gelation of the caseins. In spite of the fact that calcium and orthophosphate are the main minerals in milk and are generally present in dairy products, little was known about their combined impact on caseins in aqueous solution. Clearly, in order to have a better control of milk processing and to formulate more sophisticated dairy products it is necessary to understand the interactions between casein, calcium and orthophosphate especially in the concentrated protein regime. This was the objective of the investigation presented in this thesis.

The thesis consists of 9 chapters and a general conclusion:

Chapter 1 gives a review of the literature on the structure and behaviour casein micelles and sodium caseinate in aqueous solution.

Chapter 2 presents the materials and methods used in the research.

Chapter 3 resumes the principle results that are described in more detail in chapters 4-9 in the form of journal articles.

Chapters 4-8 contain articles that have been published (4, 5, 6, and 8) or submitted for publication (7). The manuscript given in Chapter 9 is not yet fully finished.

Chapter 4 describes the effect of calcium ions on the properties of dense caseinate solutions.

Chapter 5 shows the effect of monovalent salts on the rheology of dense caseinate solutions and the calcium binding capacity of caseinate.

Chapter 6 describes the dissociation of casein micelles after addition of soldium caseinate due to calcium chelation.

Chapter 7 reports on the combined effects of orthophosphate and calcium ions on the organization of sodium caseinate in aqueous solution.

Chapter 8 describes the properties of dense suspensions of sodium caseinate and casein micelles using ^{31}P MAS NMR as a function of the pH.

Chapter 9 describes heat-induced gelation of casein micelle and sodium caseinate suspensions at different pH.

Chapter 1. Bibliography.

1.1. Casein, presentation and description.

1.1.1. Casein, the main protein in milk.

Milk is a biologic fluid secreted by mammals to nutritionally fulfill their neonate. Milk is composed of several molecules divided into three phases. The aqueous phase (serum) contains lactose and small soluble molecules (proteins, peptides, urea, vitamins and minerals). The oil phase is composed of small dispersed fat droplets. Finally, the colloidal phase is composed of caseins and minerals.

Milk contains 3.2-3.5wt% proteins divided into caseins (80% of the total protein content) and whey proteins (20 %).

1.1.2. The individual caseins.

The caseins, are present in mainly four different types, encoded by different genes (Ginger and Grigor 1999) : α_{s1}, α_{s2}, β and κ-casein in proportions 4 : 1: 3.5 : 1.5, respectively (Dalgleish 2011) and are not homogeneously distributed in milk (see Table 1). Indeed, about 90 % of the caseins (Davies and Law 1983) are associated into a colloidal complex with calcium and phosphate which is called the “casein micelle” although it is not a true micelle as it is nowadays commonly understood (Fox and Brodkorb 2008). A small fraction of the casein is in the serum (Davies and Law 1983; Rollema 1992) in dynamic equilibrium with the micelles depending on pH and temperature (Davies and Law 1983).

Table 1. Distribution of the caseins between the serum and micellar phases of bovine milk at pH 6.7 and 20°C, from Davies and Law (1983).

Component	Micellar phase ($g.L^{-1}$)	Serum phase ($g.L^{-1}$)
α_{s1}-casein	10.9	0.7
α_{s2}-casein	3	0.1
β-casein	9	1.3
κ-casein	2.9	0.5
Calcium	0.8	0.4
Phosphate	0.9	1.1
Citrate	0.1	1.8

Caseins are considered as rheomorphic proteins because they do not need any particular secondary structure to fulfill their biological function (Holt 1999; Horne 2002; de Kruif and Holt 2003). They exhibit an open and flexible conformation (Swaisgood 1993; Holt 1999; de Kruif and Holt 2003; Thorn, Ecroyd et al. 2015) with little secondary structure compared to other proteins. They can have different conformations depending on their environment (Horne 2002).

Caseins are all phosphoproteins that present an amphiphilic nature (Swaisgood 1993; Horne 2009) but differ in their primary amino acid sequence and the rate of post-translational glycosylation and phosphorylation (de Kruif and Holt 2003; Holland 2009). Their main properties are summarized in the Table 2. Phosphorylation occurs on serine (SerP) or threonine residues that are associated in clusters within the primary sequence (Table 2). The phosphorylated sites are located in the polar regions and are preferred fixation sites for calcium. α_{s1}, α_{s2} and β are well-known Ca-sensitive caseins whereas κ-casein is Ca-insensitive, because it contains only one or two phosphorylated amino acids.

Table 2. Main properties of the different caseins (Huppertz 2013).

Casein	α_{s1}	α_{s2}	β	κ
Proportion	30-40%	10-15%	25-40%	10-15%
Number of aminoacid	199	207	209	169
Molecular weight ($g.mol^{-1}$)	23 600	25 200	24 000	19 000
Charge at pH 6.6	-22	-15	-12	-3
pI (phosphorylated)	4.42	4.95	4.65	5.6
Number of phosphorylations	8-9	10-13	5	1-2
Number of Cys	0	2	0	2

1.1.3. The casein micelle.

1.1.3.1. Description of the casein micelle. Individual caseins are associated into a colloidal complex called the casein micelle. The casein micelle is composed of caseins (94% wt) and minerals (6 %wt), mainly calcium and phosphate (Horne 2009; Dalgleish 2011; O'Mahony and Fox 2013).

Casein micelles are spherical particles with a high polydispersity (de Kruif and Holt 2003; Bouchoux, Gesan-Guiziou et al. 2010; Dalgleish 2011; O'Mahony and Fox 2013). Sizes range between 50-600nm (Horne 2006; O'Mahony and Fox 2013) with a number average diameter of 100nm (Horne 2006). Casein micelles are highly hydrated (3.7 g H_2O/g protein) (Mc Mahon, Brown 1984). Their main characterics are summarized in Table 3.

Table 3. Physico-chemical properties of casein micelles (Mc Mahon, Brown 1984).

Parameter	Values
Average Diameter	130-160 nm
Surface	8.10^{-10} cm^2
Volume	$2.1.10^{-15}$ mL
Density (hydrated)	1.0632
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 $mL.g^{-1}$
Water content	63%
Number of micelle per L of milk	10^{17}-10^{19}

1.1.3.2. Biosynthesis and biological function of the casein micelles. The synthesis of the casein micelles occurs in the epithelial cells in the vesicles of the mammary gland (Farrell, Malin et al. 2006). Individual caseins are synthetized in the ribosomes. The proteins, associated into 10nm particles, migrate to the Golgi apparatus where they are phosphorylated (Farrell, Malin et al. 2006). This organelle contains the calcium phosphate and allows the formation of the casein micelle (Farrell, Malin et al. 2006).

Fast growing animal species contain higher protein, calcium and phosphate contents in their milk than slow growing species (de Kruif and Holt 2003; Farrell, Malin et al. 2006). High amounts of calcium present in the casein micelles are essential for transport of high quantities of proteins with a reduced viscosity that allows milks to be secreted (Holt and Hukins 1991; Farrell, Malin et al. 2006). The evident function of the casein micelle would be to feed the neonate. However, in comparison with other proteins, caseins are constituted of aminoacids with a rather low nutritional value. A major role of the casein micelle could therefore be a chaperone function (Holt, Carver et al. 2013) in order to transport high contents of calcium and phosphate while preventing calcification of the mammary gland in a way that also avoids formation of amyloid fibrils (Holt, Carver et al. 2013; Thorn, Ecroyd et al. 2015).

1.1.3.3. Structure of the casein micelle. The determination of the casein micelle was debated over 60 years and reviewed many times (Schmidt 1982; McMahon and Brown 1984; Walstra 1990; Holt 1992; Rollema 1992; Holt and Horne 1996; Walstra 1999; Horne 2002; de Kruif and Holt 2003; Farrell, Malin et al. 2006; Qi 2007; Fox and Brodkorb 2008; Horne 2009; Dalgleish 2011; McMahon and Oommen 2013; O'Mahony and Fox 2013). Globally, two main models have been proposed to describe the casein micelle. If the models agree on the presence of κ-casein at the surface of the micelle and the role of calcium phosphate in maintaining the integrity, they mainly diverged concerning the internal structure of the casein micelle. The first model described the casein micelle as an assemblage of subunits called submicelles. These submicelles contain or are crosslinked via granules of calcium phosphate (Schmidt 1982; McMahon and Brown 1984; Walstra 1990; Rollema 1992; Walstra 1999). The κ-casein at the surface is due to repartition of κ-rich submicelles at the periphery of the micelle and κ-poor submicelles in the core. This model was supported by electron microscopy technique, but many artefacts due to preparation were identified (Holt, Carver et al. 2013; McMahon and Oommen 2013; O'Mahony and Fox 2013). It was also unclear by what mechanisms a submicelle could be rich or poor in κ-casein. There is still no clear evidence of the existence of submicelles so this model is largely discredited today. The second model proposed by Holt, describes the casein micelle as a network of proteins in which particles of calcium phosphate are randomly dispersed (Figure 1). The calcium phosphate forms nanoclusters with a diameter of about 2nm (Pyne and McGann 1960; McGann, Kearney et al. 1983; Marchin, Putaux et al. 2007) surrounded by the phosphorylated serine (SerP) residues of the Ca-sensitive caseins into a colloidal calcium phosphate (CCP) (Holt 1997). In this model, the κ-casein with no affinity to calcium are located mainly at the periphery. This model was corroborated by recent progress in the techniques of electron microscopy, SANS and SAXS. Electron-dense regions have been identified within the micelles that could correspond to nanoclusters. The structure factor obtained from scattering techniques can be theoretically reproduced by this model (De Kruif, Huppertz et al. 2012), showing a shoulder at $q \approx 0.35 nm^{-1}$ that corresponds to the inverse characteristic distance between the nanoclusters (de Kruif, Van Iersel et al. 1985; Smyth, Clegg et al. 2004; Marchin, Putaux et al. 2007; Bouchoux, Gesan-Guiziou et al. 2010; De Kruif, Huppertz et al. 2012; Ingham, Erlangga et al. 2015).

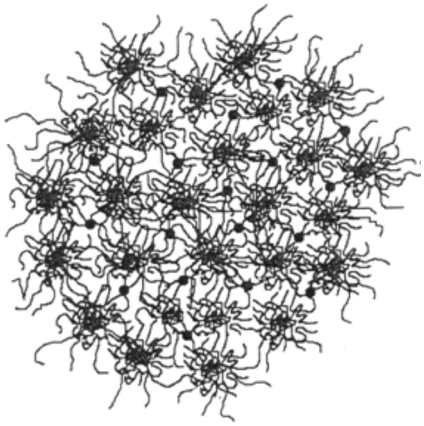

Figure 1. Structure of the casein micelle in the nanocluster model (Holt 1992).

Ameliorations to this model were recently proposed. The first nuance in the Holt model that mostly focused on the interactions between protein and calcium phosphate, was to point out the importance of protein-protein interactions (Figure 2) that can also stabilize the micelle through hydrophobic interactions and electrostatic repulsions (Horne 2002; Horne 2009). The second nuance concerns the protein network. Holt considered an homogeneous protein matrix but recent work (McMahon and Oommen 2008; Bouchoux, Gesan-Guiziou et al. 2010; Dalgleish 2011; Trejo, Dokland et al. 2011) describes the micelle as constituted of rather a heterogeneous matrix with inclusion of water channels, which was supported by SAXS and EM experiments (McMahon and Oommen 2008; Bouchoux, Gesan-Guiziou et al. 2010; Trejo, Dokland et al. 2011).

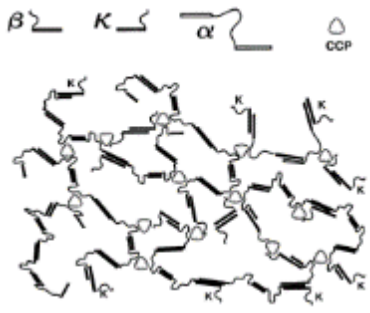

Figure 2. Dual bonding model of the casein micelle (Horne 2002). The caseins are associated together through hydrophobic interactions and with calcium phosphate through their polar region.

1.1.3.4. External surface structure of the casein micelle. The size of the micelles was found to be inversely proportional to the quantity of κ-casein (Davies and Law 1983; Horne 2006). This empirical observation was explained by the fact that κ-casein is preferentially located at the surface of the casein micelle (Holt and Horne 1996; de Kruif and Holt 2003; Horne 2006). κ-casein is not bound to calcium phosphate but to the other caseins through its hydrophobic region (Horne 2002). The hydrophilic region of κ-casein is free and protrudes 5-10nm from the surface of the micelle in the form of a hairy layer (Holt and Horne 1996) or a polyelectrolyte brush (de Kruif and Zhulina 1996; Tuinier and de Kruif 2002; de Kruif and Holt 2003) that inhibits aggregation of the micelles. The distribution of the κ-casein at the surface of the micelle is probably inhomogeneous (Dalgleish 1998) and would explain the heterogeneities observed by microscopy (Dalgleish, Spagnuolo et al. 2004; Ouanezar, Guyomarc'h et al. 2012).

1.2. The minerals of milk.

Milk is rich in salts divided into cations Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} and anions Cl^{-}, PO_4^{2-}, Cit^{-} (Pyne 1962; Gaucheron 2005), see Table 4.

Table 4. Mineral composition of milk (Gaucheron 2005).

Mineral	Concentration (mmol.kg^{-1})
Calcium	26-32
Magnesium	4-6
Inorganic phosphate	19-23
Citrate	7-11
Sodium	17-28
Potassium	31-43
Chloride	22-34

Calcium and phosphate are at oversaturated concentrations in milk. However, most of the calcium and half of phosphorus is contained in the casein micelles which prevents calcium phosphate precipitation. Milk is in thermodynamic equilibrium (Figure 3) with minerals split

into diffusible (within the serum) and non-diffusible (within the casein micelles) (Holt 1997). The mineral equilibrium between these two phases is essential because it contributes to the structure and the stability of the casein micelle.

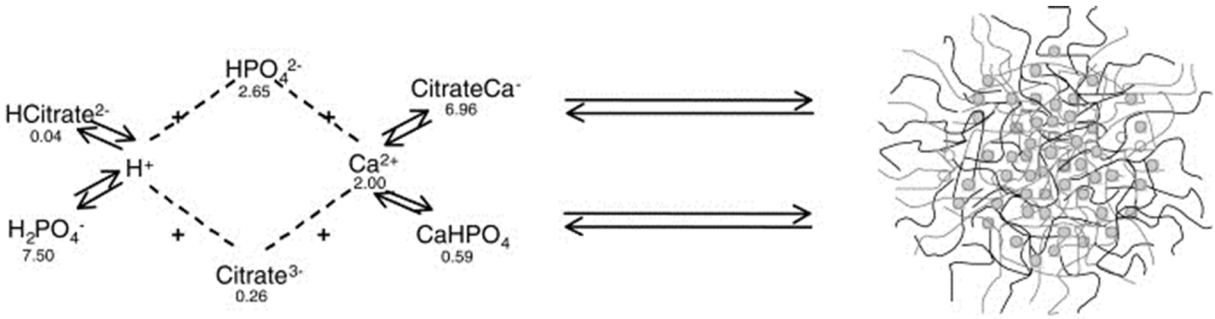

Figure 3. Mineral equilibrium between the serum and micellar phase. The concentrations of free salts are indicated in mM in the figure (Philippe, Le Graët et al. 2005).

1.2.1. The diffusible ions of milk.

The diffusible ions correspond to the fraction of minerals in the serum of milk. Serum can be obtained either by dialysis, ultrafiltration, renneting or ultracentrifugation of milk (Pyne 1962). Some minerals of the diffusible phase are able to exchange with the colloidal phase (Zhang and Aoki 1996). In this phase, ion pairs are formed according to the solubility and affinity between anions and cations (Mekmene, Le Graët et al. 2009). The composition can be calculated in good agreement with experimentally determined values using the Holt nanocluster model (Holt 2004; Mekmene, Le Graët et al. 2009).

The serum contains almost all free Na^+, K^+ and Cl^- (Holt 1985). About one third of calcium and half of the phosphate ions are in the serum essentially in the form of complexes, but also as free ions (Holt 1985).

1.2.2. The non-diffusible ions and nature of colloidal calcium phosphate.

The non-diffusible mineral fraction of milk consists mainly of Ca^{2+} and PO_4^{2-} and a small amount of Mg^{2+} and Cit^-. If some Ca^{2+} and Mg^{2+} are directly bound to the proteins, most of the minerals are located in the nanoclusters.

Nanoclusters of calcium phosphate of 2.5nm in diameter are homogeneously distributed within the micelle (McGann, Buchheim et al. 1983; McGann, Kearney et al. 1983; Marchin, Putaux et al. 2007) and are fundamental for the integrity of the micelle in milk. Calcium phosphate can be enzymatically extracted from micelles to give a micellar calcium phosphate (MCP) similar to CCP (Holt 1985; Holt 1997) composed of calcium and phosphate and to a lesser extent of Mg (1/3 of total Mg) (Pyne 1962) and citrate (10% of total Cit) (Pyne 1962). In the nanoclusters, the ratios Mg/P_i=0.044 and Cit=0.097 (Holt 1982) are relatively low. The mineral composition of the non-diffusible phase has been studied in order to determine the structure of CCP nanoclusters that is still unclear. Nanoclusters are described as a loose crystalline form (Horne 2009), brushite (Holt 1982; Holt, Hasnain et al. 1982), apatite type tricalcium phosphate containing citrate type $3Ca_3(PO_4)_2CaH$ (Pyne and McGann 1960; McMahon and Brown 1984), rather basic amorphous (Schmidt 1982; McGann, Buchheim et al. 1983; McGann, Kearney et al. 1983) type $Ca_9(PO_4)_6$ (Schmidt 1982) or acid calcium phosphate similar to non-ordered brushite (Lyster, Mann et al. 1984; Holt, Van Kemenade et al. 1989) or influenced by presence of magnesium or citrate (Holt, Van Kemenade et al. 1989). The ratio of Ca to orthophosphate (P_i) is 1.6 in MCP (Pyne and McGann 1960; Holt, Hasnain et al. 1982; McGann, Buchheim et al. 1983; McGann, Kearney et al. 1983). This ratio corresponds to a basic calcium phosphate but if the phosphate of SerP (P_o) is also considered, the ratio becomes $Ca/(P_i + P_o)$=1, which is closer to that of acid calcium phosphate (Holt, Van Kemenade et al. 1989). Holt (Holt 1997) showed that SerP was part of the CCP.

1.3. The casein micelle : a stable structure?

The casein micelle can be considered as a stable entity because it can withstand up a certain extent treatments such as freezing, drying, heating, compaction by osmotic pressure or

ultracentrifugation, addition of calcium or ethanol (Holt and Horne 1996; de Kruif 1999). Nevertheless, under certain conditions the casein micelles can be destabilized or dissociated. Destabilization leads to aggregation and even to gelation of the casein micelle suspensions, whereas dissociation leads to formation of smaller particles.

1.3.1. Origin of the stability of the casein micelle.

The casein micelle may be modelled as an associative colloid covered with a surface composed of the hydrophilic part of the κ-casein (de Kruif and Zhulina 1996; de Kruif 1999; Tuinier and de Kruif 2002). The surface is considered as a hairy layer (Holt and Horne 1996) or salted polyelectrolyte brush in native milk conditions (de Kruif and Zhulina 1996). The layer provides stabilization to the micelle through steric repulsion (Holt and Horne 1996) but there is also Van der Waals attraction and electrostatic repulsion between the micelles, see Figure 4 (Tuinier and de Kruif 2002). The steric stabilization is lost when the charge density or the chain density (de Kruif and Zhulina 1996) of the layer is reduced (Holt and Horne 1996; de Kruif 1999).

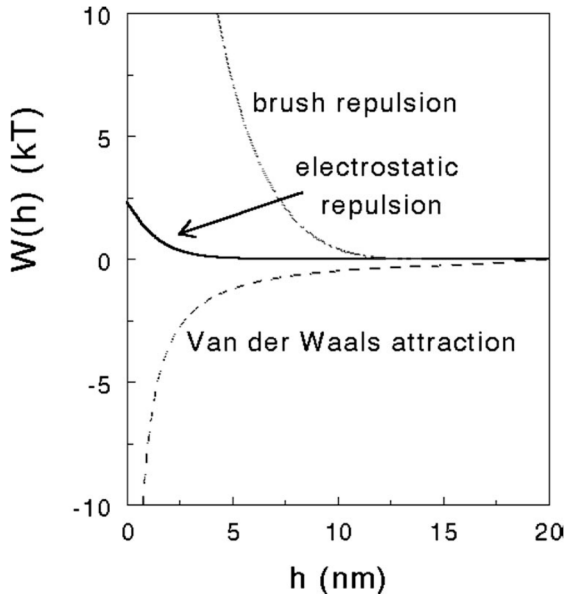

Figure 4. Interaction potential as a function of the distance between two casein micelles at pH 6.7 due to steric and electrostatic repulsion, and Van der Waals attraction (Tuinier and de Kruif 2002).

1.3.2. Factors affecting the stability of casein micelles.

1.3.2.1. Effect of rennet. Rennet is composed of two enzymes pepsin and the more active chymosin. Action of rennet releases the hydrophilic segment κ-casein from the micelle in the form of soluble para-κ-casein (Bringe and Kinsella 1987; Horne and Banks 2004). The remaining paracasein micelle is devoid of its stabilizing brush and coagulates (Walstra 1990; de Kruif 1999; Tuinier and de Kruif 2002). Enzymatic coagulation of casein micelles is the first step for producing cheese.

1.3.2.2. Effect of acidification. Acidification of milk is one critical step for production of dairy products such as cheese or yogurts. Acidification can be either biological through bacterial fermentation or chemical through the addition of acid.

Many studies showed that acidification of milk leads to an increase of the minerals in the serum phase of milk due to solubilization of CCP (Pyne 1962; Visser, Miniham et al. 1986; Dalgleish and Law 1989; Le Graët and Brulé 1993; Famelart, Lepesant et al. 1996; Law 1996; Singh, Roberts et al. 1996; Famelart, Le Graët et al. 1999; Silva, Piot et al. 2013; Koutina, Knudsen et al. 2014). At pH 5.2, all the micellar phosphorus is solubilized implying that the calcium bound to the phosphate is also solubilized. However, all the micellar calcium is completely solubilized only at pH 4.8, implying that part of the calcium is dircectly bound to the proteins (Le Graët and Brulé 1993; Singh, Roberts et al. 1996). Decreasing the pH approaching pI=4.6, leads to reduction of the net charge of the proteins. Precipitation of caseins starts at pH~5 (Kim and Kinsella 1989; Famelart, Lepesant et al. 1996). However, down to this pH, the casein micelles do not show significant changes in their size (Visser, Miniham et al. 1986; Kim and Kinsella 1989; Vreeman, Van Markwijk et al. 1989; de Kruif and Holt 2003; Anema, Lowe et al. 2004; Marchin, Putaux et al. 2007; McMahon, Du et al. 2009) because the caseins are still associated in the micelle through increased hydrophobic interactions. Dissociation of a small fraction of casein micelles (Dalgleish and Law 1988; Famelart, Lepesant et al. 1996; Singh, Roberts et al. 1996) occurs at low pH and especially at low temperatures (Dalgleish and Law 1988) at which hydrophobic interactions are weaker. This dissociation seems to affect more the smallest casein

micelles (McMahon, Du et al. 2009) and is increased by the presence of salts (Famelart, Le Graët et al. 1999) due to screening of electrostatic interaction.

Demineralized and with the κ-casein brush collapsed on the surface of the micelle, the structure of the micelles after acidification is thus not the same as in milk. Acidification leads to aggregation and gelation of the casein micelle suspensions (Lucey and Singh 1997) (Figure 5). The structure of aggregates formed by acidified micelles is described as fractal with a fractal dimension $D_f \approx 2.3$ (Bremer, Bijsterbosch et al. 1990; Lucey and Singh 1997). The aggregation process was also described in terms of the adhesive hard sphere model (De Kruif 1997; Lucey and Singh 1997; de Kruif 1999).

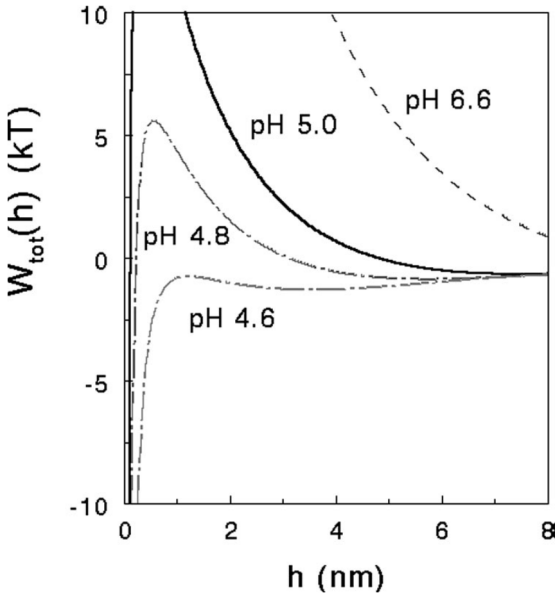

Figure 5. Interaction potential as a function of the distance between two casein micelles at different pH due to steric and electrostatic repulsion and Van der Waals attraction (Tuinier and de Kruif 2002).

1.3.2.3. Effect of the temperature. On cooling, β-casein is reversibly removed from the micelle (de Kruif and Holt 2003), which does not change its size (Marchin, Putaux et al. 2007) but can cause changes of the structure (Yazdi, Corredig et al. 2014) and some mineral solubilization (Holt 1985; Gaucheron 2005). The fact that β-casein can be removed from the micelle at low temperatures suggests that it is bound mainly by hydrophobic interactions. The effect of heating on the stability of the casein micelles is complex, resulting in a combination of different reactions depending on the conditions (presence of lactose, whey protein, etc.) (Fox 1981). At physiological conditions, heating milk at T<100°C does not cause severe modifications to the

structure of the casein micelles (Livney, Corredig et al. 2003; O'Connell and Fox 2003; Gaucheron 2005). It only induces some reversible changes in the mineral equilibrium of milk with reversible precipitation of calcium phosphate (Pyne 1962; Holt 1995; Gaucheron 2005; Lucey and Horne 2009). When the temperature exceeds 100°C, micelle stability is lost leading to milk coagulation (Fox 1981; O'Connell and Fox 2000; Singh 2004) and (κ-) casein solubilisation (Aoki, Suzuki et al. 1974; Walstra 1990). At such high temperatures caseins are dephosphorylated (Fox 1981; Meisel, Andersson et al. 1991; Singh 2004; Gaucheron 2005) and calcium phosphate salts precipitates (Fox 1981; Singh 2004) in the forms of hydroxyapatite which is different from the original CCP (Fox 1981).

1.3.2.4. Effect of ionic strength. Addition of sodium chloride induces a decrease of the pH of milk and an increase of the diffusible calcium (Ramet, El Mayda et al. 1983; Le Graët and Brulé 1993; Le Ray, Maubois et al. 1998; Famelart, Le Graët et al. 1999; Huppertz and Fox 2006) and orthophosphate (Famelart, Le Graët et al. 1999). Release of calcium from the micelle is presumably caused by exchanges between sodium and cations bound to the micelle and by reduction in the ion activity coefficient of calcium phosphate (Gaucheron 2005; Huppertz and Fox 2006). Increase of calcium and phosphorus in the aqueous phase is accompanied by an increase in the nitrogen content (Famelart, Le Graët et al. 1999).

Addition of calcium ions modifies the salt distribution between the diffusible and non-diffusible fractions (Jeurnink and De Kruif 1995; Gastaldi, Pellegrini et al. 1996; Gaucheron 2005). When calcium ions are added to casein micelles, the micelle undergoes some structural changes which do not affect their average diameter (Udabage, McKinnon et al. 2000; Philippe, Gaucheron et al. 2003; Philippe, Le Graët et al. 2005). A small amount of calcium complexes with inorganic phosphate (Udabage, McKinnon et al. 2000; Philippe, Gaucheron et al. 2003; Philippe, Le Graët et al. 2005) and citrate in the serum and integrates the micellar phase (Philippe, Gaucheron et al. 2003; Philippe, Le Graët et al. 2005). Some caseins contained in the serum become insoluble, and are probably also incorporated (Le Ray, Maubois et al. 1998; Philippe, Gaucheron et al. 2003; Philippe, Le Graët et al. 2005) in the micellar phase causing the micelles to become denser (Lin, Leong et al. 1972; Philippe, Gaucheron et al. 2003). At 120mM (Famelart, Le Graët et al. 1999), most of the calcium remains in the aqueous phase and the structure of the casein micelles is only moderately influences. However, further increasing the

calcium concentration induces aggregation and precipitation of casein (Lin, Leong et al. 1972; Udabage, McKinnon et al. 2000).

1.3.3. Dissociation.

Despite the stability of the casein micelles, they can be dissociated in smaller particles. These particles were historically called submicelles in reference to the submicelle model, which is now abandoned.

1.3.3.1. Dissociation methods. The casein micelle can be dissociated by high pressure treatments (Gaucheron, Famelart et al. 1997; Huppertz, Kelly et al. 2002; Huppertz, Kelly et al. 2006; Orlien, Knudsen et al. 2006; Knudsen and Skibsted 2010) and use of chaotropes such as urea (Morr 1967; Holt 1998).

Dissociation also occurs when the CCP is removed by dialysis (Ono, Dan et al. 1978; Stothart and Cebula 1982; Aoki, Yamada et al. 1988; Holt 1998) or calcium chelatants such as EDTA (Lin, Leong et al. 1972; Griffin, Lyster et al. 1988; Udabage, McKinnon et al. 2000; Marchin, Putaux et al. 2007; Pitkowski, Nicolai et al. 2007), citrate (Udabage, McKinnon et al. 2000; Mizuno and Lucey 2005; de Kort, Minor et al. 2011; Kaliappan and Lucey 2011) or phosphates of different nature (Mizuno and Lucey 2005; Pitkowski, Nicolai et al. 2007; de Kort, Minor et al. 2011).

There is a fraction of calcium and phosphate that can be removed without disrupting the micelle (Griffin, Lyster et al. 1988), but further removal causes dissociation. After addition of a calcium chelatant, a fraction of the dissociation occurs very rapidly and is followed by slow further dissociation (Pitkowski, Nicolai et al. 2007). The size of the undissociated casein micelles is unchanged (Griffin, Lyster et al. 1988; Udabage, McKinnon et al. 2000) and there is no preferentially solubilization of the individual caseins molecules (Griffin, Lyster et al. 1988; Pitkowski, Nicolai et al. 2007), suggesting an “all-or-nothing” dissociation process. This means that a micelle is either completely dissociated or remains intact (Pitkowski, Nicolai et al. 2007).

After dissociation, depending on the environment, casein molecules are associated into particles with a radius of about 10nm (Ono, Dan et al. 1978; Pepper and Farrell 1982; Panouillé, Nicolai

et al. 2004; Pitkowski, Nicolai et al. 2007) and a molar mass of about $2.10^5 g.mol^{-1}$ (Panouillé, Nicolai et al. 2004) through hydrophobic interactions. Suspensions of these submicelles suspensions often contain a low weight fractions of fat residues (<1%) that together with a small fraction of the proteins form particles with a radius of about 60nm that strongly contributes to the light scattering intensity (Panouillé, Nicolai et al. 2004; HadjSadok, Pitkowski et al. 2008).

1.3.3.2. Aggregation of casein micelles dissociated with polyphosphate. Submicelles which are obtained after dissociation of casein micelles with polyphosphate are unstable. They aggregate and can even form gels with time probably due to the presence of calcium ions complexed with phosphates (Panouillé, Nicolai et al. 2004; Panouillé, Durand et al. 2005; Pitkowski, Nicolai et al. 2007). The aggregation process strongly depends on ionic strength, pH and temperature with an activation energy of $90kJ.mol^{-1}$ independently of the casein concentration (Pitkowski, Nicolai et al. 2007). The aggregates observed by cryo-TEM are composed of branched chains of small spheres with a diameter of about 20 nm and the size of the aggregate increases with time (Panouillé, Durand et al. 2005).

1.4. Sodium caseinate

1.4.1. Structure of the sodium caseinate particles

Sodium caseinate is obtained by acidification that dissolves calcium phosphate and precipitates caseins. The calcium phosphate is removed by washing and the caseins are resolubilized by increasing the pH to neutral with NaOH (Mulvihill and Fox 1989; Southward 1989).

Aqueous suspensions of sodium caseinate have been extensively studied. In the presence of salts, the caseins are associated into particles with a hydrodynamic radius of about 10nm (Kumosinski, Pessen et al. 1988; Chu, Zhou et al. 1995; Farrer and Lips 1999; HadjSadok, Pitkowski et al. 2008), resulting from the association of about 15 casein molecules (HadjSadok, Pitkowski et al. 2008). The size of the caseinate particles depends on environmental conditions (pH, T°C, ionic strength) (HadjSadok, Pitkowski et al. 2008).

1.4.2. Behaviour of dense sodium caseinate suspensions.

Suspensions of sodium caseinate (NaCas) are translucent at low protein concentration and opalescent at high protein concentrations due to residual fat/protein globules (HadjSadok, Pitkowski et al. 2008; Bouchoux, Ventureira et al. 2015).

The viscosity of sodium caseinate suspensions increases sharply with increasing protein concentration for $C>100 g.L^{-1}$ due to jamming of the caseinate particles, but does not diverge because the particles can interpenetrate (Pitkowski, Durand et al. 2008; Bouchoux, Debbou et al. 2009; Loveday, Rao et al. 2010). Jamming also leads to an increase of the relaxation rate of the protein protons as measured with ^{1}H NMR relaxometry (Bouchoux, Schorr et al. 2012). Dense suspensions of sodium caseinate exhibit visco-elastic behavior (Pitkowski, Durand et al. 2008; Loveday, Rao et al. 2010).

1.4.3. Influence of temperature, pH.

Caseinate suspensions are relatively stable when heated or cooled, but the viscosity of the suspensions reversibly decreases when the temperature increases. This was attributed to a decrease of the effective volume fraction (Pitkowski, Durand et al. 2008) of the particles or a decrease of the solvent quality (de Kruif, Bhatt et al. 2015). High temperature treatments (140°C) lead to irreversible changes of NaCas like protein hydrolysis or aggregation (Chu, Zhou et al. 1995; Hustinx, Singh et al. 1997). When $pH \leq 5.2$, the acid precipitation of NaCas occurs (Lucey and Singh 1997; HadjSadok, Pitkowski et al. 2008; Morales, Martinez et al. 2015) due to charge neutralization.

1.4.4. Influence of calcium ions.

1.4.4.1. Effect of calcium. As was already mentioned, calcium binds specifically to casein and the affinity of caseins for calcium depends on the number of SerP. Phosphoserines are organized into clusters in the polypeptide sequence. Individual α_{s1}-, α_{s2}-, β- and κ-caseins contain respectively 3, 2, 1 and 0 clusters in their sequence (de Kruif and Holt 2003; Swaisgood 2003).

However, SerP are not the only sites for calcium fixation aspartic and glutamic acid are also candidates for calcium binding (Dickson and Perkins 1971; Bingham, Farrell et al. 1972; Ono, Kaminogawa et al. 1976).

κ-casein is soluble in the presence of even a large amount of calcium as it does not contain a SerP cluster (Ono, Yahagi et al. 1980). α_{s1}-, α_{s2}- and β-caseins are soluble in the presence of calcium only up to a low critical concentration above which the solutions become turbid (Parker and Dalgleish 1977) and caseins precipitate (Bingham, Farrell et al. 1972; Ono, Kaminogawa et al. 1976; Imade, Sato et al. 1977; Dalgleish, Paterson et al. 1981; Parker and Dalgleish 1981; Aoki, Toyooka et al. 1985; Baumy and Brulé 1988). The critical concentration that induces precipitation depends on the affinity for calcium and is in the order $\alpha_{s2} > \alpha_{s1} > \beta > \kappa$ (Dickson and Perkins 1971; Imade, Sato et al. 1977). However, the precipitation process depends more on the ability of the proteins to form hydrophobic interactions or hydrogen bounds, rather than differences in their calcium binding process (Parker and Dalgleish 1981; Aoki, Toyooka et al. 1985; Rollema 1992; Swaisgood 2003; Huppertz 2013). α_{s2} is the most calcium sensitive casein and thus precipitates at lower calcium concentrations than the other caseins (Aoki, Toyooka et al. 1985). Binding of calcium to α_{s1}-casein was reported to follow a two-stage kinetic process involving the creation of a transient monomer-octamer equilibrium that initiates the precipitation (Parker and Dalgleish 1977; Dalgleish, Paterson et al. 1981). Calcium binding to α_{s1}-casein is therefore described to occur in a sequential process involving binding of calcium through phosphoserines (or carboxyl residues) followed by changes of the conformation and hydration of the proteins. Then, these structural changes induce a new degree of association, and additional changes of the nature of bonding and solvatation (Holt, Parker et al. 1975;

Swaisgood 1993; Swaisgood 2003; Huppertz 2013). The reaction was described as first exothermic and then endothermic with increasing the calcium concentration (Swaisgood 1993; Swaisgood 2003; Huppertz 2013). β-casein is less sensitive (Farrell Jr, Kumosinski et al. 1988) than α_s-caseins to calcium (Parker and Dalgleish 1981). Suspensions of β-caseins were found to form micron sized aggregates after addition of calcium (Figure 6).

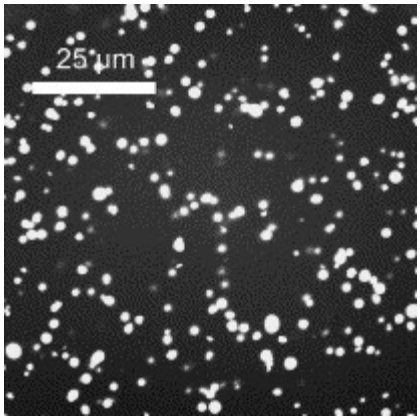

Figure 6. CLSM image of β-casein solution (C=1g.L^{-1}, 10 mM $CaCl_2$) after 30 min at 35 °C (Dauphas, Mouhous-Riou et al. 2005).

In a sodium caseinate suspension, each type of casein is present in different proportions. Addition of more than a critical concentration of calcium leads to an increase of the turbidity of the sodium caseinate suspensions and to the precipitation of casein (Zittle and Dellamonica 1958; Guo, Campbell et al. 2003; Pitkowski, Nicolai et al. 2009). The increase of the turbidity is due to the formation of large particles. The fraction of sedimentable caseins (Zittle and Dellamonica 1958; Guo, Campbell et al. 2003; Pitkowski, Nicolai et al. 2009) and the viscosity of the suspensions were found to decrease while the turbidity increased (Carr, Munro et al. 2002) with increasing concentration of calcium. Calcium was found to change the fluorescence behaviour of the caseinate (Cuomo, Ceglie et al. 2011; Lopez, Cuomo et al. 2013) suggesting structural changes in the environment of aromatic residues of the casein. The calcium binding is marked by a sudden increase of the scattering intensity of caseinate suspensions analyzed by DLS (Chu, Zhou et al. 1995) at a critical concentration of calcium. As for individual caseins, up to a critical concentration of calcium, the size of caseinate particles remains unchanged (Chu, Zhou et al. 1995; Pitkowski, Nicolai et al. 2009) implying that caseins can bind calcium up to a certain extent without changing their degree of association.

1.4.4.2. Effect of temperature. Calcium induced precipitation increases with increasing the temperature (Farrell Jr, Kumosinski et al. 1988; Cuomo, Ceglie et al. 2011; Lopez, Cuomo et al. 2013) due to strengthening of the hydrophobic interactions. Decreasing the temperature has the opposite effect and β-casein, the most hydrophobic casein, is not precipitated by calcium at 1°C (Parker and Dalgleish 1981; Farrell, Kumosinski et al. 1988).

1.4.4.3. Effect of pH and ionic strength. Calcium binding decreases with protonation of the phosphoserines when the pH is decreased. As a consequence, casein binds less Ca^{2+}. Nevertheless caseinate precipitates at lower Ca^{2+} due to its lower charge density (Zittle and Dellamonica 1958; Dickson and Perkins 1971; Baumy and Brulé 1988).

Addition of NaCl leads to an increase of the free Ca^{2+} concentration by competition for Na^{+} (Dickson and Perkins 1971). However, a large excess of NaCl is necessary in order to reduce significantly the calcium binding (Huppertz 2013). For example at pH 7.4, 0.5M of NaCl are necessary to unbind 2.5mM of Ca^{2+} in a α_{s1}-casein solution (Dickson and Perkins 1971).

References

Anema, S. G., E. K. Lowe, et al. (2004). "Effect of pH on the viscosity of heated reconstituted skim milk." *International Dairy Journal* 14(6): 541-548.

Aoki, T., H. Suzuki, et al. (1974). "Formation of soluble casein in whey protein-free milk heated at high temperature." *Milchwissenschaft* 29(10): 589-594.

Aoki, T., K. Toyooka, et al. (1985). "Role of phosphate groups in the calcium sensitivity of α_{s2}-casein." *Journal of Dairy Science* 68(7): 1624-1629.

Aoki, T., N. Yamada, et al. (1988). "Dissociation during dialysis of casein aggregates cross-linked by colloidal calcium phosphate in bovine casein micelles." *Journal of Dairy Research* 55: 180-195.

Baumy, J. J. and G. Brulé (1988). "Effect of pH and ionic strength on the binding of bivalent cations to β-casein." *Le Lait* 68: 409-418.

Bingham, E. W., H. M. Farrell, et al. (1972). "Properties of dephosphorylated α_{s1}-casein. Precipitation by calcium ions and micelle formation." *Biochemistry* 11(13): 2450-2454.

Bouchoux, A., B. Debbou, et al. (2009). "Rheology and phase behavior of dense casein micelle dispersions." *The Journal of Chemical Physics* 131: 165106.

Bouchoux, A., G. Gesan-Guiziou, et al. (2010). "How to squeeze a sponge: casein micelles under osmotic stress, a SAXS study." *Biophysical Journal* 99(11): 3754-3762.

Bouchoux, A., D. Schorr, et al. (2012). "Molecular mobility in dense protein systems: an investigation through ^{1}H NMR relaxometry and diffusometry." *The Journal of Physical Chemistry* B 116(38): 11744-11753.

Bouchoux, A., J. Ventureira, et al. (2015). "Structural heterogeneity of milk casein micelles: a SANS contrast variation study." *Soft Matter* 11(2): 389-399.

Bremer, L. G. B., B. H. Bijsterbosch, et al. (1990). "On the fractal nature of the structure of acid casein gels." *Colloids and Surfaces* 51: 159-170.

Bringe, N. A. and J. E. Kinsella (1987). "Forces involved in the enzymatic and acidic coagulation of casein micelles." *Developments in Food Proteins* 5: 159-194.

Carr, A. J., P. A. Munro, et al. (2002). "Effect of added monovalent or divalent cations on the rheology of sodium caseinate solutions." *Journal of Dairy Research* 12: 487-492.

Chu, B., Z. Zhou, et al. (1995). "Laser light scattering of model casein solutions: effects of high temperature." *Journal of Colloid and Interface Science* 170(1): 102-112.

Cuomo, F., A. Ceglie, et al. (2011). "Temperature dependence of calcium and magnesium induced caseinate precipitation in H_2O and D_2O." *Food Chemistry* 126(1), 8-14.

Dalgleish, D. G. (1998). "Casein micelles as colloids: Surface structures and stabilities." *Journal of Dairy Science* 81(11): 3013-3018.

Dalgleish, D. G. (2011). "On the structural models of bovine casein micelles—review and possible improvements." *Soft Matter* 7(6): 2265-2272.

Dalgleish, D. G. and A. J. Law (1988). "pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins." *Journal of Dairy Research* 55(04): 529-538.

Dalgleish, D. G. and A. J. R. Law (1989). "pH-induced dissociation of bovine casein micelles. II mineral solubilization and its relation to casein release." *Journal of Dairy Research* 56: 727-735.

Dalgleish, D. G., E. Paterson, et al. (1981). "Kinetics of aggregation of α_{s1}-casein / Ca^{2+} mixtures: charge and temperature effects." *Biophysical Chemistry* 13: 307-314.

Dalgleish, D. G., P. A. Spagnuolo, et al. (2004). "A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy." *International Dairy Journal* 14: 1025-1031.

Dauphas, S., N. Mouhous-Riou, et al. (2005). "The supramolecular organisation of β-casein: effect on interfacial properties." *Food Hydrocolloids* 19(3): 387-393.

Davies, D. T. and A. J. R. Law (1983). "Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature." *Journal of Dairy Research* 50: 67-75.

de Kort, E., M. Minor, et al. (2011). "Effect of calcium chelators on physical changes in casein micelles in concentrated micellar casein solutions." *International Dairy Journal* 21(12): 907-913.

de Kruif, C. and C. Holt (2003). Casein micelle structure, functions and interactions. *Advanced Dairy Chemistry, Vol. 1, Proteins*. 3rd ed. P. F. Fox and P. L. H. McSweeney. New York, NY, USA, Kluwer Academic/Plenum Publishers: 233-276.

de Kruif, C. G. (1997). "Skim milk acidification." *Journal of Colloid and Interface Science* 185(1): 19-25.

de Kruif, C. G. (1999). "Casein micelle interactions." *International Dairy Journal* 9: 183-188.

de Kruif, C. G., T. Huppertz, et al. (2012). "Casein micelles and their internal structure." *Advances in Colloid and Interface Science* 171: 36-52.

de Kruif, C. G., E. M. F. Van Iersel, et al. (1985). "Hard sphere colloidal dispersions: Viscosity as a function of shear rate and volume fraction." *The Journal of Chemical Physics* 83: 4717.

de Kruif, C. G. and E. B. Zhulina (1996). "κ-casein as a polyelectrolyte brush on the surface of casein micelles." *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 117: 151-159.

de Kruif, C. G. K., H. Bhatt, et al. (2015). "Rheology of caseinate fractions in relation to their water holding capacity." *Food Hydrocolloids* 51: 503-511.

Dickson, I. R. and D. J. Perkins (1971). "Studies on the interactions between purified bovine caseins and alkaline-earth-metalions." *Biochemistry. J* 124: 235-240.

Famelart, M., Y. Le Graet, et al. (1999). "Casein micelle dispersions into water, NaCl and $CaCl_2$ physicochemical characteristics of micelles and rennet coagulation." *International Dairy Journal* 9(3): 293-297.

Famelart, M., F. Lepesant, et al. (1996). "pH-Induced physicochemical modifications of native phosphocaseinate suspensions: Influence of aqueous phase." *Le Lait* 76(5): 445-460.

Farrell, H. M., T. F. Kumosinski, et al. (1988). "Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization." *Archives of Biochemistry and Biophysics* 265: 146-158.

Farrell, H. M., E. L. Malin, et al. (2006). "Casein micelle structure: what can be learned from milk synthetis and structural biology." *Current Opinion in Colloid & Interface Science* 11(2-3): 135-147.

Farrell Jr, H. M., T. F. Kumosinski, et al. (1988). "Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization." *Archives of Biochemistry and Biophysics* 265: 146-158.

Farrer, D. and A. Lips (1999). "On the self-assembly of sodium caseinate." *International Dairy Journal* 9: 281-286.

Fox, P. F. (1981). "Heat-Induced Changes in Milk Preceding Coagulation." *Journal of Dairy Science* 64(11): 2127-2137.

Fox, P. F. and A. Brodkorb (2008). "The casein micelle: Historical aspects, current concepts and significance." *International Dairy Journal* 18(7): 677-684.

Gastaldi, E., O. Pellegrini, et al. (1996). "Functions of added calcium in acid milk coagulation." *Journal of Food Science* 59: 310-312.

Gaucheron, F. (2005). "The minerals of milk." *Reproduction Nutrition Development* 45(4): 473-484.

Gaucheron, F., M.-H. Famelart, et al. (1997). "Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk." *Food Chemistry* 59(3): 439-447.

Ginger, M. R. and M. R. Grigor (1999). "Comparative aspects of milk caseins." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 124(2): 133-145.

Griffin, M. C. A., R. L. J. Lyster, et al. (1988). "The disaggregation of calcium-depleted casein micelles." *European Journal of Biochemistry* 174: 339-343.

Guo, M., B. E. Campbell, et al. (2003). "Casein precipitation equilibria in the presence of calciums ions and phosphates." *Colloids and Surfaces B: Biointerfaces* 29: 297-307.

HadjSadok, A., A. Pitkowski, et al. (2008). "Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering." *Food Hydrocolloids* 22: 1460-1466.

Holland, J. W. (2009). "Post-translational modifications of caseins." *Milk proteins: From Expression to Food*: 107-132.

Holt, C. (1982). "Inorganic constituents of milk III. The colloidal calcium posphate of cow milk." *Journal of Dairy Research* 49: 29-38.

Holt, C. (1985). The milk salts: their secretion, concentrations and physical chemistry. *Developments in Dairy Chemistry Springer*: 143-181.

Holt, C. (1992). "Structure and stability of bovine casein micelles." *Advances of Protein Chemistry* 43: 63-151.

Holt, C. (1995). "Effect of heating and cooling on the milk salts and their interaction with casein." *Bulletin- International Dairy Federation* 9501(Spec. iss.): 105-133.

Holt, C. (1997). The milk salts and their interaction with casein. *Advanced Dairy Chemistry Volume 3*, Springer: 233-256.

Holt, C. (1998). "Casein micelle substructure and calcium phosphate interactions studied by sephacryl column chromatography." *Journal of Dairy Science* 81: 2994-3003.

Holt, C. (1999). Casein structure and casein-calcium phosphate interactions. 25. *International Dairy Congress, 2: Dairy Science and Technology*, Aarhus (Denmark), 21-24 Sep 1998, IDF.

Holt, C. (2004). "An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk." *Journal of European Biophysics* 33: 421-434.

Holt, C., J. A. Carver, et al. (2013). "Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods." *Journal of Dairy Science* 96(10): 6127-6146.

Holt, C., S. S. Hasnain, et al. (1982). "Structure of bovine milk calcium phosphate determined by x-ray absorption spectroscopy." *Biochimica et Biophysica Acta (BBA)* - General Subjects 719(2): 299-303.

Holt, C. and D. S. Horne (1996). "The hairy casein model: evolution of the concept and its implications for dairy technology." *Netherlands Milk & Dairy Journal* 50: 85-111.

Holt, C. and D. W. L. Hukins (1991). "Structural analysis of the environment of calcium ions in crystalline and amorphous calcium phosphates by X-ray absorption spectroscopy and a hypothesis concerning the biological function of the casein micelle." *International Dairy Journal* 1(3): 151-165.

Holt, C., T. G. Parker, et al. (1975). "The thermochemistry of reactions between α_{s1}-casein and calcium chloride." *Biochemica et Biophysica Acta* 379: 638-644.

Holt, C., M. J. J. M. Van Kemenade, et al. (1989). "Composition and structure of micellar calcium phosphate." *Journal of Dairy Research* 56: 411-416.

Horne, D. S. (2002). "Casein structure, self assembly and gelation." *Current Opinion in Colloid and Interface Science* 7: 456-461.

Horne, D. S. (2006). "Casein micelle structure: models and muddles." *Current Opinion in Colloid & Interface Science* 11(2): 148-153.

Horne, D. S. (2009). Casein micelle structure and stability, *Milk proteins From Expression to Food*, Academic Press.

Horne, D. S. and J. M. Banks (2004). "Rennet-induced coagulation of milk." *Cheese: chemistry, physics and microbiology* 1: 47-70.

Huppertz, T. (2013). Chemistry of the Caseins. *Advanced Dairy Chemistry*. New-York, NY, USA, Springer: 135-160.

Huppertz, T. and P. F. Fox (2006). "Effect of NaCl on some physico-chemical properties of concentrated bovine milk." *International Dairy Journal* 16(10): 1142-1148.

Huppertz, T., A. L. Kelly, et al. (2006). "Disruption and reassociation of casein micelles under high pressure." *Journal of Dairy Research* 73(03): 294-298.

Huppertz, T., A. L. Kelly, et al. (2002). "Effects of high pressure on constituents and properties of milk." *International Dairy Journal* 12(7): 561-572.

Hustinx, J. C. A., T. K. Singh, et al. (1997). "Heat-induced hydrolysis of sodium caseinate." *International Dairy Journal* 7: 207-212.

Imade, T., Y. Sato, et al. (1977). "Interaction of calcium ion with bovine caseins." *Agricultural and Biological Chemistry* 41(11): 2131-2137.

Ingham, B., G. D. Erlangga, et al. (2015). "Solving the mystery of the internal structure of casein micelles." *Soft Matter* 11(14): 2723-2725.

Jeurnink, T. J. M. and K. G. De Kruif (1995). "Calcium concentration in milk in relation to heat stability and fouling." *Netherlands Milk and Dairy Journal* 49: 151-151.

Kaliappan, S. and J. Lucey (2011). "Influence of mixtures of calcium-chelating salts on the physicochemical properties of casein micelles." *Journal of Dairy Science* 94(9): 4255-4263.

Kim, B. Y. and J. E. Kinsella (1989). "Effect of temperature and pH on the coagulation of casein." *Milchwissenschaft* 44(10): 622-625.

Knudsen, J. C. and L. H. Skibsted (2010). "High pressure effects on the structure of casein micelles in milk as studied by cryo-transmission electron microscopy." *Food Chemistry* 119(1): 202-208.

Koutina, G., J. C. Knudsen, et al. (2014). "Temperature effect on calcium and phosphorus equilibria in relation to gel formation during acidification of skim milk." *International Dairy Journal* 36(1): 65-73.

Kumosinski, T. F., H. Pessen, et al. (1988). "Determination of the quaternary structural states of bovine casein by small-angle x-ray scattering: submicellar and micellar forms." *Archives of Biochemistry and Biophysics*. 266(2): 548-561.

Law, A. J. R. (1996). "Effects of heat treatment and acidification on the dissociation of bovine casein micelles." *Journal of Dairy Research* 63: 35-48.

Le Graet, Y. and G. Brulé (1993). "Les équilibres minéraux du lait: influence du pH et de la force ionique." *Le Lait* 73(1): 51-60.

Le Ray, C., J.-L. Maubois, et al. (1998). "Heat stability of reconstituted casein micelle dispersions: changes induced by salt addition." *Le Lait* 78(4): 375-390.

Lin, S., S. Leong, et al. (1972). "Effect of calcium ion on the structure of native bovine casein micelles." *Biochemistry* 11(10): 1818-1821.

Livney, Y. D., M. Corredig, et al. (2003). "Influence of thermal processing on the properties of dairy colloids." *Current Opinion in Colloid & Interface Science* 8(4): 359-364.

Lopez, F., F. Cuomo, et al. (2013). "Effects of solvent and alkaline earth metals on the heat-induced precipitation process of sodium caseinate." *Food Chemistry* 136(1): 266-272.

Loveday, S. M., M. A. Rao, et al. (2010). "Rheological behavior of high concentration sodium caseinate dispersions." *Journal of Food Science* 75(2): N30-N35.

Lucey, J. A. and D. S. Horne (2009). Milk salts: Technological significance. *Advanced Dairy Chemistry*, Springer: 351-389.

Lucey, J. A. and H. Singh (1997). "Formation and physical properties of acid milk gels: a review." *Food Research International* 30(7): 529-542.

Lyster, R. L. J., S. Mann, et al. (1984). "Nature of micellar calcium phosphate in cows' milk as studied by high-resolution electron microscopy." *Biochimica et Biophysica Acta (BBA)*-General Subjects 801(2): 315-317.

Marchin, S., J.-L. Putaux, et al. (2007). "Effects of the environmental factors on the casein micelle structure studied by cryo-transmission electron microscopy and small angle x-ray scattering/ultra small-angle x-ray scattering." *Journal of Chemical Physics* 126: 126-135.

McGann, T. C. A., W. Buchheim, et al. (1983). "Composition and ultrastructure of calcium phosphate-citrate complexes in bovine milk systems." *Biochimica et Biophysica Acta (BBA)*-General Subjects 760(3): 415-420.

McGann, T. C. A., R. D. Kearney, et al. (1983). "Amorphous calcium phosphate in casein micelles of bovine milk." *Calcified tissue international* 35(1): 821-823.

McMahon, D. J. and R. J. Brown (1984). "Composition, structure and integrity of casein micelles: a review." *Journal of Dairy Science* 67: 499-512.

McMahon, D. J., H. Du, et al. (2009). "Microstructural changes in casein supramolecules during acidification of skim milk." *Journal of Dairy Science* 92(12): 5854-5867.

McMahon, D. J. and B. S. Oommen (2008). "Supramolecular structure of the casein micelle." *Journal of Dairy Science* 91(5): 1709-1721.

McMahon, D. J. and B. S. Oommen (2013). Casein micelle structure, functions, and interactions. *Advanced Dairy Chemistry*, Springer: 185-209.

Meisel, H., H. B. Andersson, et al. (1991). "Heat-induced changes in casein-derived phosphopeptides." *Zeitschrift für Ernährungswissenschaft* 30(3): 227-232.

Mekmene, O., Y. Le Graet, et al. (2009). "A model for predicting salt equilibria in milk and mineral-enriched milks." *Food Chemistry* 116(1): 233-239.

Mizuno, R. and J. Lucey (2005). "Effects of emulsifying salts on the turbidity and calcium-phosphate–protein interactions in casein micelles." *Journal of Dairy Science* 88(9): 3070-3078.

Morales, R., M. J. Martinez, et al. (2015). "Impact of casein glycomacropeptide on sodium caseinate self-assembly and gelation." *International Dairy Journal* 49, 30-36.

Morr, C. V. (1967). "Effect of oxalate and urea upon ultracentrifugation properties of raw and heated skimmilk casein micelles." *Journal of Dairy Science* 50(11): 1744-1751.

Mulvihill, D. M. and P. F. Fox (1989). Caseins and caseinate manufacture. *Developments in Dairy Chemistry*. P. F. Fox. London, UK, Elsevier Applied Science. 4: 97-130.

O'Connell, J. E. and P. F. Fox (2000). "The two-stage coagulation of milk proteins in the minimum of the heat coagulation time-pH profile of milk: effect of casein micelle size." *Journal of Dairy Science* 83(3): 378-386.

O'Connell, J. E. and P. F. Fox (2003). Heat-induced coagulation of milk. *Advanced Dairy Chemistry - Proteins*, Springer: 879-945.

O'Mahony, J. A. and P. F. Fox (2013). Milk proteins: introduction and historical aspects. *Advanced Dairy Chemistry*, Springer: 43-85.

Ono, T., H. T. Dan, et al. (1978). "Dissociation of bovine casein micelles by dialysis." *Agricultural and Biological Chemistry* 42: 1063-1064.

Ono, T., S. Kaminogawa, et al. (1976). "A study on the binding of calcium ions to α_{s1}-casein." *Agricultural and Biological Chemistry* 40(9): 1717-1723.

Ono, T., M. Yahagi, et al. (1980). "The binding of calcium to κ-casein and para κ-casein." *Agricultural and Biological Chemistry* 44(7): 1499-1503.

Orlien, V., J. C. Knudsen, et al. (2006). "Dynamics of casein micelles in skim milk during and after high pressure treatment." *Food Chemistry* 98: 513-521.

Ouanezar, M., F. Guyomarc'h, et al. (2012). "AFM imaging of milk casein micelles: evidence for structural rearrangement upon acidification." *Langmuir* 28(11): 4915-4919.

Panouillé, M., D. Durand, et al. (2005). "Aggregation and gelation of micellar casein particles." *Journal of Colloid and Interface Science*: 85-93.

Panouillé, M., T. Nicolai, et al. (2004). "Heat induced aggregation and gelation of casein submicelles." *International Dairy Journal* 14: 297-303.

Parker, T. G. and D. G. Dalgleish (1977). "The use of light scattering and turbidity measurements to study the kinetics of extensively aggregating proteins: α_{s1}-casein." *Biopolymers* 16: 2533-2547.

Parker, T. G. and D. G. Dalgleish (1981). "Binding of calcium ions to bovine β-casein." *Journal of Dairy Research* 48: 71-76.

Pepper, L. and H. M. Farrell (1982). "Interactions leading to formation of casein submicelles." *Journal of Dairy Science* 65: 2259-2266.

Philippe, M., F. Gaucheron, et al. (2003). "Physicochemical characterization of calcium-supplemented skim milk." *Le Lait* 83(1): 45-59.

Philippe, M., Y. Le Graët, et al. (2005). "The effects of different cations on the physicochemical characteristics of casein micelles." *Food Chemistry* 90(4): 673-683.

Pitkowski, A., D. Durand, et al. (2008). "Structure and dynamical mechanical properties of suspensions of sodium caseinate." *Journal of Colloid and Interface Science* 326(1): 96-102.

Pitkowski, A., T. Nicolai, et al. (2007). "Dynamical mechanical characterization of gelling micellar casein particles." *Journal of Rheology* 52: 971.

Pitkowski, A., T. Nicolai, et al. (2007). "Scattering and turbidity study of the dissociation of casein by calcium chelation." *Biomacromolecules* 9(1): 369-375.

Pitkowski, A., T. Nicolai, et al. (2009). "Stability of caseinate solutions in the presence of calcium." *Food Hydrocolloids* 23(4): 1164-1168.

Pyne, G. T. (1962). "Section C. Dairy Chemistry: Some aspects of the physical chemistry of the salts of milk." *Journal of Dairy Research* 29(01): 101-130.

Pyne, G. T. and T. C. A. McGann (1960). "The colloïdal calcium phosphate of milk. II influence of citrate." *Journal of Dairy Research* 27: 9-17.

Qi, P. X. (2007). "Studies of casein micelle structure: the past and the present." *Le Lait* 87(4-5): 363-383.

Ramet, J. P., E. El-Mayda, et al. (1983). "Influence of salting of reconstituted milk on curdling by rennet." *Journal of Texture Studies* 14(1): 11-19.

Rollema, H. S. (1992). "Casein association and micelle formation." *Advanced Dairy Chemistry 1*: 111-140.

Schmidt, D. G. (1982). Association of caseins and casein micelle structure. *Developments in Dairy Chemistry*. P.F.Fox. London, Applied Science Publishers, Essex. I: 61-86.

Silva, N. N., M. Piot, et al. (2013). "pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties." *Food Hydrocolloids* 32(2): 322-330.

Singh, H. (2004). "Heat stability of milk." *International Journal of Dairy Technology* 57(2-3): 111-119.

Singh, H., M. S. Roberts, et al. (1996). "Acid-induced dissociation of casein micelles in milk: Effects of heat treatment." *Journal of Dairy Science* 79(8): 1340-1346.

Smyth, E., R. A. Clegg, et al. (2004). "A biological perspective on the structure and function of caseins and casein micelles." *International Journal of Dairy Technology* 57(2-3): 121-126.

Southward, C. R. (1989). Used of casein and caseinates. *Developement in Dairy Chemistry-4*. F. P. Fox. London, Elsiever Applied Science: 173-244.

Stothart, P. H. and D. J. Cebula (1982). "Small-angle neutron scattering study of bovine casein micelles and sub-micelles." *J. Mol. Biol.* 160: 391-395.

Swaisgood, H. E. (1993). "Review and update of casein chemistry." *Journal of Dairy Science* 76: 3054-3061.

Swaisgood, H. E. (2003). Chemistry of the caseins. *Advanced Dairy Chemistry - Proteins*, Springer: 139-201.

Thorn, D. C., H. Ecroyd, et al. (2015). "Casein structures in the context of unfolded proteins." *International Dairy Journal* 46: 2-11.

Trejo, R., T. Dokland, et al. (2011). "Cryo-transmission electron tomography of native casein micelles from bovine milk." *Journal of Dairy Science* 94(12): 5770-5775.

Tuinier, R. and C. G. de Kruif (2002). "Stability of casein micelles in milk." *Journal of Chemical Physics* 117(3): 1290-1295.

Udabage, P., I. R. McKinnon, et al. (2000). "Mineral and casein equilibria in milk: effects of added salts and calcium-chelating agents." *Journal of Dairy Research* 67: 361-370.

Visser, J., A. Miniham, et al. (1986). "Effects of pH and temperature on the milk salt system." *Netherlands Millk and Dairy Journal* 40: 351-368.

Vreeman, H. J., B. W. Van Markwijk, et al. (1989). "The structure of casein micelles between pH 5.5 and 6.7 as determined by light scattering, electron microscopy and voluminosity experiments." *J. Dairy Res.* 56(3): 463-470.

Walstra, P. (1990). "On the stability of casein micelles." *Journal of Dairy Science* 73(8): 1965-1979.

Walstra, P. (1999). "Casein sub-micelles: do they exist?" *International Dairy Journal* 9: 189-192.

Yazdi, S. Y., M. Corredig, et al. (2014). "Studying the structure of β-casein-depleted bovine casein micelles using electron microscopy and fluorescent polyphenols." *Food Hydrocolloids* 42: 171-177.

Zhang, Z. P. and T. Aoki (1996). "Behaviour of calcium and phosphate in bovine casein micelles." *International Dairy Journal* 6: 769-780.

Zittle, C. A. and E. S. Dellamonica (1958). "Binding of calcium to casein: influence of pH and calcium and phosphate concentrations." *Archives of Biochemistry and Biophysics* 76: 342-353.

Chapter 2. Materials and Methods.

2.1. Materials

2.1.1. Caseinate powders.

For almost all of the experiments the sodium caseinate and calcium caseinate powders were provided by Armor Protéines, Saint-Brice-en-Coglès (France). The main composition of the powders given by the provider is reported in Table 1.

Table 1. Chemical composition of sodium and calcium caseinate powders.

	Sodium caseinate	Calcium caseinate
Proteins (TNC *6.38) (g/100g powder)	91	91
Minerals (g/100g powder)	4	4
Sodium (mg/100g powder)	1300	30
Calcium (mg/100g powder)	50	1300
Total phosphorus (mg/100g powder)	700	700
Fat (g/100g powder)	0.7	0.7

The NaCas powder, Lactonat EN, Lactoprot, Kaltenkirchen (Germany) was also used and had the same chemical composition as the NaCas powder from Armor Protéines.

2.1.2. Casein micelle powder.

The casein micelle powder used in this study is native phosphocaseinate (NPCP) kindly provided from INRA STLO in Rennes. This powder is obtained after tangential microfiltration (0.14µm) and diafiltration of skim milk as described in Schuck, Piot et al. 1994.

The chemical composition of this powder is given in Table 2.

Table 2. Chemical composition of native phosphocaseinate powders.

Proteins (TNC *6.38) (g/100g powder)	83
Minerals (g/100g powder)	8.72
Calcium (mg/100g powder)	2585
Total phosphorus (mg/100g powder)	1700
Inorganic phosphorus	~60% of total phosphorus
Sodium (mg/100g powder)	0

2.1.3. Preparation of the solutions.

The solutions were prepared by dissolving the powder into ultrapure water containing 3mM of sodium azide used to prevent bacterial contamination.

The powders were dispersed into water by magnetic stirring at 20°C. The homogenization was completed for caseinate by heating for ~30 minutes at 80°C and for NPCP by heating overnight at 50°C. Solutions of NaCas were stable for months, but the micelles disintegrated slowly even in dense suspensions and therefore NPCP solutions were conserved at most one week at 200g.L^{-1}.

Salts solutions ($CaCl_2$, NaCl, NaH_2PO_4/Na_3PO_4 1:1, etc.) were prepared by dissolving the powders in ultrapure water (NaN_3, 3mM). Aliquots of stock solutions of concentrated salt solutions were added as required under vigorous stirring.

2.2. Methods.

2.2.1. Ultracentrifugation.

Most of the time, centrifugations were carried out at 20°C with an Allegra 64R centrifuge (Beckman Coulter, USA) at 50 000*g* rotor speed during 2 h.

Sedimentation of particles depends on:

- the centrifugal force,
- the particle density,
- the solution density (when the solution density is greater than the particle density, the particle velocity is negative : creaming).
- the friction coefficient which related to viscosity, particle shape, etc….

When sodium caseinate solutions were centrifuged, a top layer was formed containing residual fat globules together with less than 5% of the proteins.

2.2.2. Protein content.

The protein content (C) was determined by measuring the absorbance at 280nm (A_{280}) into cuves of width L. The solutions were diluted to about 1g.L^{-1}. The extinction coefficient (Ɛ) was found to be 0.85 L.g^{-1}.cm^{-1} by comparing with the concentration determined with the Kjeldhal method. C was calculated using the Beer-Lambert relation:

$$C = \frac{A_{280}}{\varepsilon \cdot l} \quad (1)$$

2.2.3. Turbidity measurements.

The turbidity was determined over a range of wavelengths where the proteins did not absorb the light (400-1100 nm). For an isotropic system, the turbidity is given by:

$$\tau = \ln\left(\frac{I_0}{I}\right)\frac{1}{L} \quad (2)$$

with I_0 the intensity of the incident light and I the intensity of the transmitted light.

Experimentally, the turbidity was deduced from the measured absorbance (A): τ = A.ln(10)/L.

Turbidity measurements were done as a function of the wavelength in rectangular air tight cells using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France). Different path lengths

(1, 2, 5 and 10mm) were used depending on the turbidity of the samples in order to avoid saturation. Measurements were done at different temperatures that were controlled using a thermostat bath.

2.2.4. Rheology.

Flow measurements. During flow measurements the sum of forces (F) applied on a sample induces a stress (σ) which is parallel to the surface of the sample, leading to a deformation γ. When γ is derived with time, the shear rate ($\dot{\gamma}$) is obtained and the shear rate dependent viscosity is derived from the relation

$$\eta=\sigma/\dot{\gamma} \tag{3}$$

Oscillatory measurements. Oscillatory shear measurements were used to characterize the mechanical properties of the materials as a function of the shear stress (σ) and the oscillation frequency. A sinusoidal shear stress was imposed with frequency (ω): $\sigma = \sigma_0.\sin(\omega.t)$, and resulting deformation (γ) of the material was measured: $\gamma = \gamma_0.\sin(\omega.t+\delta)$, where δ is the phase shift. At low stresses in the so-called linear response regime $\gamma_0 \propto \sigma_0$ and the in phase and out of phase deformation are characterized by the storage (G') and the loss modulus (G"), respectively:

$$G'(\omega) = \frac{\sigma_0}{\gamma_0} \sin\delta \tag{4}$$

$$G''(\omega) = \frac{\sigma_0}{\gamma_0} \cos\delta \tag{5}$$

For elastic solids G' is larger than G" and independent of the frequency, while for Newtonian liquids G' is smaller than G" and both moduli depend on the frequency: $G' \propto \omega^{-2}$ and $G'' \propto \omega^{-1}$. Viscoelastic materials show solid-like behavior at high frequencies and liquid -like behavior at low frequencies.

Materials. For almost all the experiments, the rheological behaviour was characterized using stress-imposed rheometer AR 2000 and ARG2 (TA Instruments, USA) with a cone-plate geometry. For samples with zero-shear viscosity $\eta<10$ Pa.s the rheological behavior was determined using another stress-imposed rheometer MCR 301 (Anton Paar, Germany) with a Couette geometry. The rheometers were equipped with a Peltier system for the control of the temperature. The samples were covered with paraffin oil to prevent water evaporation.

2.2.5. CLSM.

Conventional optical microscopy is a common technique to study colloidal suspensions. However, it often suffers from low contrast and multiple scattering from objects that are out of focus within the illuminated region.

Confocal microscopy may solve these problems. Figure shows the working principle of a Confocal Laser Scanning Microscope. With this method, the light from the laser source passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen. For scanning, the microscope uses a dichromatic mirror that reflects light shorter than a certain wavelength, but transmits light of longer wavelength. Thus the light from the laser source is reflected and scanned across the specimen in a defined focal plane, by the dichromatic mirrors. The fluoresced (higher wavelength) light from the sample passes back through the objective and is descanned by the same mirrors used to scan the sample. Before it reaches the detector, the fluoresced light passes through the pinhole which is placed in the conjugate focal (hence the term confocal) plane of the sample. The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole and hence most of this out of focus light is not detected by the photomultiplier tube and does not contribute to the resulting image. In confocal microscopy, there is never a complete image of the specimen because at any instant only one point is observed. Thus, for visualization, the detector is attached to a computer, which builds up the image, one pixel at a time. The speed of most confocal microscopes is limited by the rate at which the mirrors can scan the entire sample plane. This particular type of fluorescence microscopy, in which the objective used by the illuminating light is also used by the fluorescence light in conjunction with a dichroic mirror, is called epifluorescence.

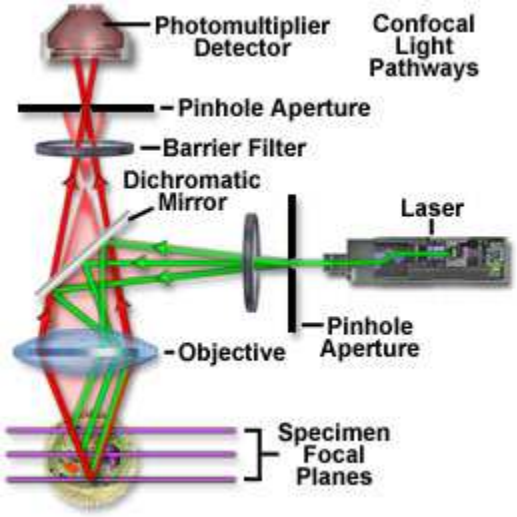

Figure 1. Basic configuration of a modern Confocal Laser Scanning Microscope.

In this study, Confocal Laser Scanning Microscopy (CLSM) was used in the fluorescence mode. Observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany). A water immersion objective lens was used HCxPL APO 63x NA=1.2 with theoretical resolution of 0.3μm in the x-y plane. Proteins were labelled with the fluorochrome rhodamine b, by adding a small amount of concentrated solution to the caseins at a final concentration of 5ppm.

2.2.6. Solid state MAS ^{31}P NMR.

Nuclear Magnetic Resonanace (NMR) is a spectroscopy technique that allows determination of the structure and the chemical environment of molecules.

This technique is based on the fact that nuclei that have a non-zero spin. ^{31}P with non zero spin nuclei and a natural abundance of 100% can be oriented in a magnetic field B_0 proportional to a resonance radiofrequency (ω).

A single pulse signal of the magnetic field is applied. This signal is influenced by the local electronic environment of the nuclei such as the presence of charged groups, polarized chemical bounds, aromatic groups, etc. The NMR signal is detected while the nuclear spins are relaxing back to their equilibrium state. The resulting free induction decay (FID) is treated by Fourier Transform. The final NMR spectrum shows the resonance (peak) corresponding to one type of nuclei as a function of the chemical shift (δ) that depends on the local environment of the nuclei.

For solids the signals are very broad and in order to improve the resolution magic angle spinning (MAS) is applied.

The NMR experiments were conducted on a Bruker Avance III 300 MHz WB spectrometer equipped with a 4 mm MAS VTN type probe head with two channels. An HR/MAS rotor was filled with about 55 µL of a casein or caseinate solution at C = 100g.L^{-}1 and spun at 3 kHz. ^{31}P direct excitation spectra were accumulated over between 128 and 1024 repetitions with a relaxation delay of 30 s, an acquisition time of 0.2 s, and a ^{1}H decoupling with reduced power (8.5 kHz) in order to avoid probe head damage during the relatively long acquisition needed for adequate sampling of relatively narrow signals. In addition, ^{1}H-^{31}P cross-polarization (CP) spectra have been acquired with 6144 repetitions with a relaxation delay of 5 s in order to characterize the broad signal from immobile phosphorus. All spectra have been normalized with respect to the H_2O ^{1}H signal in order to account for the variation in the sample volume in the HR/MAS rotor. The line width and position of the broad signal from immobile P were determined from the complementary cross-polarization spectra. The mobile signals from inorganic and organic phosphate were quantified by deconvolution and integration of the corresponding peaks in the ^{31}P direct excitation spectra after subtraction of the immobile P signal. The spectra have been referenced to an aqueous solution of H_3PO_4.

References

Schuck, P., M. Piot, et al. (1994). "Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane." *Le Lait* 74(5): 375-388.

Chapter 3. Principal Results

3.1. Behaviour of caseinate suspensions in the presence of calcium ions.

The viscosity normalized by that of water (η_{rel}) of NaCas has been determined as a function of the protein concentrations at different temperatures at pH 6.7 (Figure 1). The viscosity of NaCas increases sharply for C>80g.L^{-1} following a steep power law concentration dependence, due to jamming of protein particles. However, the viscosity decreases when the temperature increases. The decrease of the viscosity occurs in parallel with an increase of the aggregation number and R_h (HadjSadok, Pitkowski et al. 2008) of the particles with heating and is attributed to a decrease of the effective volume fraction (Pitkowski, Durand et al. 2008). However, the data obtained at different temperatures cannot be superimposed by shifting the concentrations, i.e. in terms of an effective volume fraction. This means that in the jammed state the interactions between the particles depend on the temperature.

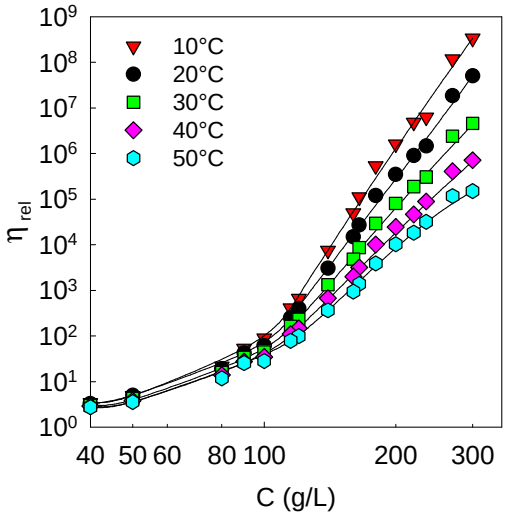

Figure 1. Concentration dependence of the relative zero shear viscosity (η_{rel}) of NaCas solutions at pH 6.7 at different temperatures indicated in the figure.

Dense suspensions of NaCas behave like viscoelastic liquids characterized by two parameters, the high frequency elastic modulus (G_e) and the terminal relaxation time (τ). τ strongly increased with decreasing temperature or increasing protein concentration probably caused by increasing of friction between the protein particles. G_e increased with increasing temperature

showing that the particles are more difficult to deform at higher temperatures. More details are given in Chapter 4.1.

When calcium ions were added to NaCas solutions, attractive interactions induced a microphase separation of the protein into dense domains (Figure 2) that caused an increase of the turbidity and a decrease the solubility.

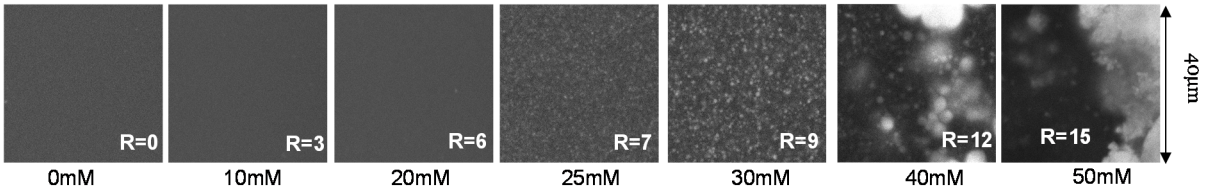

Figure 2. CLSM images (40µm×40µm) of NaCas solutions with different concentrations of $CaCl_2$ (C=80g.L^{-1}, pH 6.7, 20°C). The molar ratio (R) of Ca^{2+} per casein molecule is also indicated in the figure.

Increasing the temperature favoured phase separation so that lower amounts of Ca^{2+} were needed to decrease the solubility and increase the turbidity. However, the phenomenon is reversible as when the heated solution was cooled, the turbidity and the solubility recovered their initial values.

Addition of $CaCl_2$ strongly modified the rheological behaviour of the NaCas suspensions. On one hand, with increasing calcium concentration, the viscosity increased, because attraction between the protein particles increased. On the other hand, when increased attraction drove microphase separation a decrease of the effective volume fraction caused a drop of the viscosity. The result of these two antagonistic effects depended on the protein concentration and the temperature, see Chapter 4.1.

The terminal relaxation time increased when [Ca^{2+}] increased, but decreased when at higher [Ca^{2+}] attractive interactions became stronger. τ also decreased with increasing the temperature.

Calcium caseinate (CaCas) is a commercial ingredient which contains an intrinsic ratio of calcium ions per casein molecule (R) equal to 7. Solutions of CaCas are turbid and also exhibit microphase separation when observed with CLSM (Figure 3).

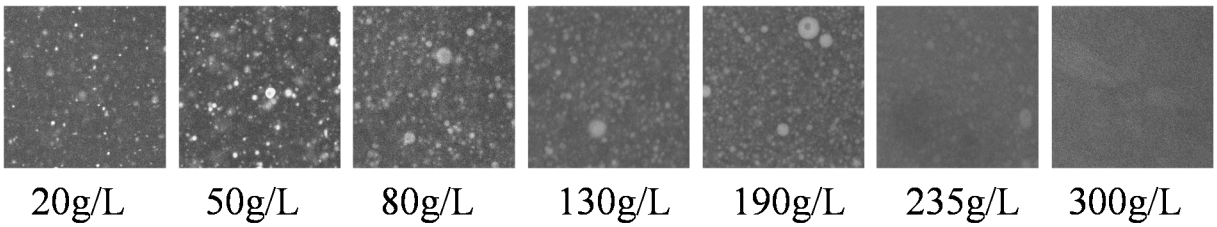

Figure 3. CLSM images (40×40µm) of calcium caseinate solutions (CaCas) as a function of the protein concentration (pH 6.7, 20°C).

Above a critical concentration of protein, which depends on the temperature, the viscosity of CaCas increases sharply, see Chapter 4.2. The viscosity of CaCas solutions strongly decreased when the temperature was increased. Figure 4 compares the viscosity of CaCas solutions as a function of the protein concentration with that of NaCas solutions. Attraction induced by calcium increased of CaCas solutions compared to NaCas solutions for C>180g.L^{-1}. At lower C, the viscosity of CaCas solutions was lower, because microphase separation decreased the effective volume fraction. Intermediate behaviour is observed for mixtures of NaCas with CaCas see Chapter 4.2. The behaviour of CaCas is determined by its Ca^{2+} content as the viscosity of the suspensions of CaCas (R=7) is very close to R=8.

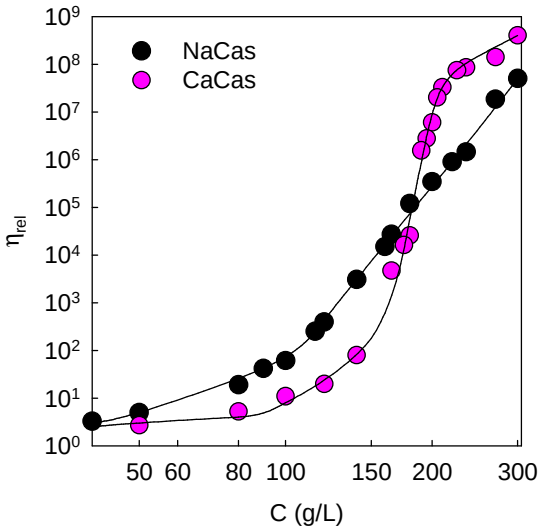

Figure 4. Concentration dependence of the relative zero shear viscosity of NaCas and CaCas solutions (20°C, pH 6.7).

3.2. Influence of monovalent salts on the properties of caseinate suspensions.

We have studied the impact of monovalent salts, mainly NaCl, on the behaviour of dense NaCas suspensions over a wide range of salt and protein concentrations at different temperatures. The results are detailed in Chapter 5.

Only little effect was observed on the viscosity when up to 250mM NaCl was added, implying that electrostatic interactions are not very important. At higher concentrations of NaCl, the viscosity strongly increased with increasing salt concentration suggesting increasing attractive interaction between the caseins caused by dehydration. The terminal relaxation time increased whereas G_e was little influenced by the addition of salt. The viscosity reversibly decreased with the temperature at all NaCl concentrations. NaCas suspensions precipitated when more than 3.5M NaCl was added, a process that is known as salting-out. The same effects were observed when NaCl was replaced by KCl.

When present in large excess, Na^+ competes with Ca^{2+} ions for binding sites on the proteins, so that increasing the amount of calcium necessary to induce microphase separation increases. This competition is marked by an increase of the calcium activity and the protein solubility with increasing the concentration of Na^+.

3.3. Dissociation of casein micelles by calcium chelation using NaCas.

Mixtures of casein micelles and NaCas have been studied over a wide range of compositions; see Chapter 6 for a detailed report. When casein micelle suspensions at low concentrations ($C=5\text{-}15 g.L^{-1}$) were mixed with NaCas, the turbidity started to decreased above a critical weight fraction of NaCas. The turbidity decreased with time in a two-stage process: a rapid decrease followed by a slower decrease. The same phenomenon was observed when casein micelles were dissociated through calcium chelation with polyphosphates or EDTA, see Chapter 1.3.3. This suggests that the dissociation of casein micelles in the mixtures occurred via calcium chelation

by NaCas. The dissociation was marked by an increase of the fraction of soluble proteins, and the fraction of calcium and phosphate that was released from the micelles. At a weight fraction of NaCas equal to 0.8 in the mixture, the casein micelles were completely solubilized. Addition of NaCas did not induce preferential solubilisation of the different types of caseins indicating an "all-or-nothing" dissociation process as was previously suggested to occur in the presence of polyphosphate. The calcium chelating capacity of NaCas is equivalent to that of polyphosphate at the same phosphate concentration. With time, the turbidity of the solutions progressively increased again implying that a reorganization of the caseins occurred in the presence of calcium and phosphate. This reorganization probably occurred in concomitance with the dissociation as for higher concentrations of casein micelles ($C>20 g.L^{-1}$) the decrease of the turbidity was less and was followed by a more rapid increase.

3.4. Effect of the combined presence of orthophosphate and calcium ions on the structure of sodium caseinate suspensions.

The mesoscopic structure of NaCas solutions containing both calcium and orthophosphate has been studied using CLSM (Figure 5) and sedimentation, see Chapter 7 for a detailed report.

As was discussed in section 3.1, in the absence of orthophosphate (P_i) increasing the concentration of calcium causes microphase separation leading to the formation of dense spherical protein particles and a decrease of the solubility. With decreasing protein concentration, the critical concentration of calcium that induces microphase separation decreased, because the microphase separation is controlled by a critical ratio of calcium per protein (R). The dense domains contain most of the α_{s1}- and α_{s2}-caseins and half of β-casein. When P_i and $CaCl_2$ was added to NaCas solutions, both were incorporated into complexes with proteins which sedimented when the solutions were centrifuged. These complexes contained all the different types of casein including κ-casein. At low concentrations of P_i large scale association of the caseins was still controlled by the number of calcium ions per protein. However, at high concentrations orthophosphate competes with protein for calcium to form calcium phosphate particles surrounded by protein layer.

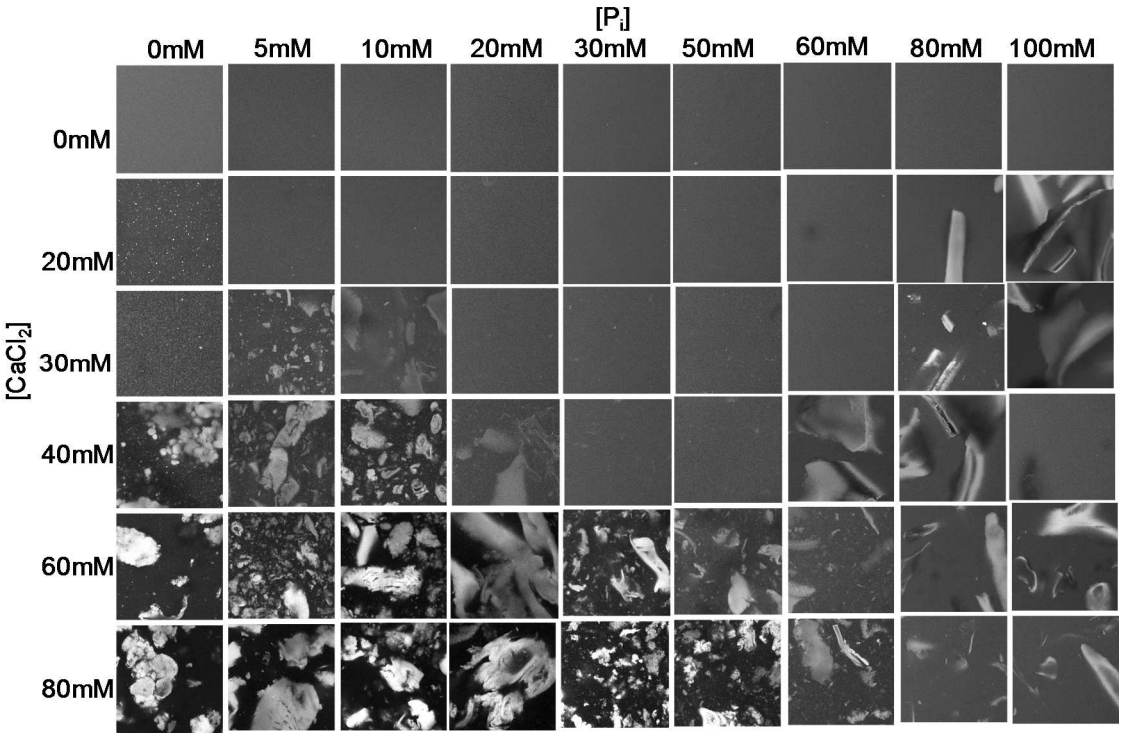

Figure 5. CLSM images (159µm×159µm) of the NaCas suspensions with calcium and orthophosphate (C=80g.L^{-1}, pH 6.7, 20°C).

The mobility of phosphorus in these systems was studied using ^{31}P NMR experiments. Addition of calcium led to a decrease of the mobility of the phosphoserines and orthophosphate. Interestingly, the amount of P_i incorporated into the complexes was found to be independent of the total amount of P_i in solution, when the latter was present in excess.

3.5. Effect of pH on the mobility of phosphorus in casein micelles and sodium caseinate in dense suspensions.

Aqueous solutions of NaCas and casein micelles were studied using ^{31}P magic angle spinning NMR technique, see Chapter 8 for a detailed report. The direct excitation spectrum of pure NaCas solutions was composed of a small peak at ~1.5ppm corresponding to a small amount of inorganic orthophosphate (P_i) and a group of three peaks corresponding to organic phosphoserines (P_o), see Figure 6. The latter results corresponded to the weighted average signal of pure α_s- and β-casein solutions whereas κ-casein that contains only one P_o does not

contribute to the NMR signal. The signal of casein micelles contains a narrow peak at 1.5ppm corresponding to the P_i signal, a broader peak corresponding to mobile P_o and a very broad peak corresponding to immobile P_o and P_i within the colloidal calcium phosphate.

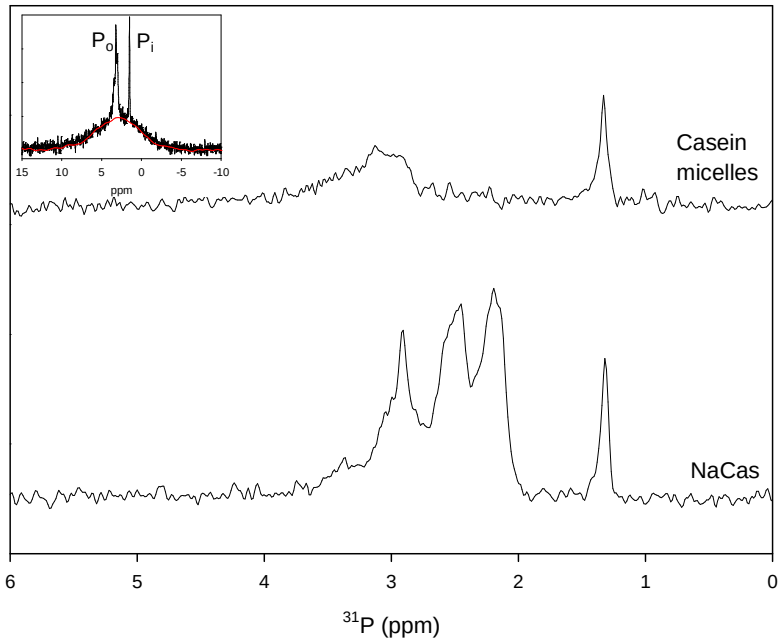

Figure 6. ^{31}P MAS NMR spectra of aqueous solutions of casein micelle and NaCas suspensions (C= 100g.L^{-1}, pH 6.8, 20°C). The inset shows the ^{31}P MAS NMR spectrum of an aqueous solution of casein micelles (C= 100g.L^{-1}, pH 6.8, 20°C) on an extended scale which better visualizes the broad signal due to immobile P. The red line represents the cross-polarization spectrum.

NaCas and casein micelles precipitate or gel below pH 5.2. When the pH was decreased to 5.2, solubilization of CCP led to an increase of the P_i signal of casein micelles solutions. The signal of both P_i and P_o shifted upfield, which was linked to the second protonation of the phosphate groups for NaCas and casein micelles. The chemical shift dependence of the pH allowed the average pKa of P_i and P_o to be determined for both systems, see Table 1.

Table 1. pKa of the phosphate groups determined by ^{31}P MAS NMR.

	P_i	P_o
Aqueous solutions	7.2*	N.D
NaCas	7.0	6.6**
Casein micelles	6.5	5.8**

* From Gaucheron 2004; ** Average values.

3.6. Heat-induced gelation of casein micelles and sodium caseinate as a function of the pH.

Heat induced aggregation and gelation of aqueous solutions of casein micelle and sodium caseinate was investigated between pH 5.2 and 6.7 over a wide range of protein concentrations (25-160g.L^{-1}), see Chapter 9 for more details. For pH≤5.2 large protein flocs were formed that precipitated. For pH≥5.3, casein micelles solutions gelled above a well-defined critical temperature (T_c) that increased with increasing pH between 25°C at pH 5.4 and 90°C and pH 6.4. T_c increased weakly when the protein concentration was decreased below C=100 g.L^{-1}. Sodium caseinate solutions also gelled when heated, but the heating temperature was not critical. For these systems the gelation rate increased progressively with increasing temperature at decreasing. For both systems heat-induced gelation is irreversible and elasticity of the gels even increases when they are cooled.

As an example, the evolution of the storage shear modulus as a function of the heating time is compared in Figure 7 for casein micelle and sodium caseinate solutions at C=160 g/L and pH 6. The casein micelle solutions gelled for T≥35°C, whereas caseinate solutions gelled only for T>60°C. Casein micelle gels were formed within a few minutes except very close to T_c, but gelation of caseinate was slow even at 80°C. The stiffness of the casein micelle gels increased steeply with increasing temperature close to T_c, but was independent of the heating temperature for $T>T_c+5$°C. The modulus of sodium caseinate gels still evolved at the end of the experiment, so it is not easy to evaluate the effect of heating temperature on the stiffness.

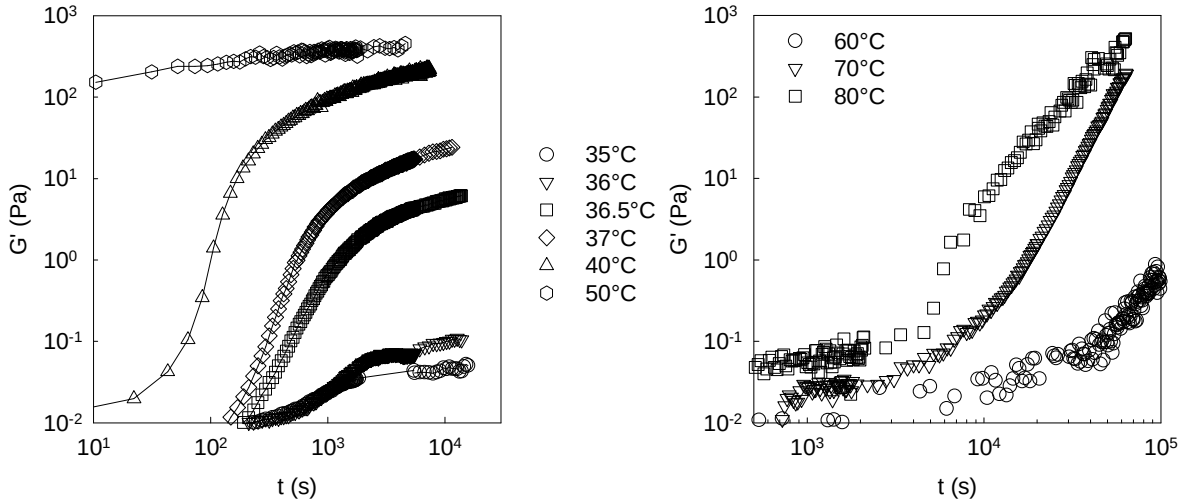

Figure 1 Storage modulus at 0.1 Hz as a function of time for casein micelle (left panel,) and sodium caseinate (right panel) suspensions at C=160g.L^{-1} at different temperatures indicated in the figure.

The observation that sodium caseinate can form a gel shows that if the electrostatic repulsion is reduced by reducing the pH, heating drives the formation of bonds between casein, which are most likely hydrophobic interactions and hydrogen bonds. The different gelling behaviour of casein micelles must be related to the different organization of the micelles in colloidal ensembles with surface layer of κ-casein and the presence of colloidal calcium phosphate.

When the pH is decreased the CCP is progressively solubilised (Dalgleish and Law 1989; Famelart, Lepesant et al. 1996) leading to an increase of the amount on minerals in the medium, but the protein skeleton of the micelle is conserved (Anema, Lowe et al. 2004).

Solubilization of CCP is also favoured by increasing the temperature (Koutina, Knudsen et al. 2014; Koutina and Skibsted 2015). It was observed that calcium phosphate induces aggregation of caseins above a critical concentration (Chapter 7). We speculate that the observed onset of gelation at a critical temperature may perhaps be related to a critical mineral concentration that induces aggregation and gelation of the micelles.

References

Anema, S. G., E. K. Lowe, et al. (2004). "Effect of pH on the viscosity of heated reconstituted skim milk." *International Dairy Journal* 14(6): 541-548.

Dalgleish, D. G. and A. J. R. Law (1989). "pH-induced dissociation of bovine casein micelles. II Mineral solubilization and its relation to casein release." *Journal of Dairy Research* 56: 727-735.

Famelart, M., F. Lepesant, et al. (1996). "pH-Induced physicochemical modifications of native phosphocaseinate suspensions: Influence of aqueous phase." *Le Lait* 76(5): 445-460.

Gaucheron, F. (2004). Minéraux et produits laitiers, *Technique & Documentation*.

HadjSadok, A., A. Pitkowski, et al. (2008). "Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering." *Food Hydrocolloids* 22: 1460-1466.

Koutina, G., J. C. Knudsen, et al. (2014). "Temperature effect on calcium and phosphorus equilibria in relation to gel formation during acidification of skim milk." *International Dairy Journal* 36(1): 65-73.

Koutina, G. and L. H. Skibsted (2015). "Calcium and phosphorus equilibria during acidification of skim milk at elevated temperature." *International Dairy Journal* 45: 1-7.

Pitkowski, A., D. Durand, et al. (2008). "Structure and dynamical mechanical properties of suspensions of sodium caseinate." *Journal of Colloid and Interface Science* 326(1): 96-102.

Chapter 4. Effect of calcium ions on the structure and rheological properties of sodium caseinate solutions.

Chapter 4.1. Slow Dynamics and Structure in Jammed Milk Protein Suspensions.

Published in *Faraday Discussions (2012),* 158(1), 325-339.

Peggy Thomar, Dominique Durand, Lazhar Benyahia, Taco Nicolai.

LUNAM Université du Maine, IMMM UMR-CNRS, 72085 le Mans Cedex 9, France.

Abstract

The dynamic mechanical properties and the structure of dense suspensions of sodium caseinate were investigated using oscillatory shear rheology and confocal laser scanning microscopy, respectively. Caseins are the most abundant milk proteins and form in the absence of calcium phosphate small star-like particles with a radius of about 10nm. The viscosity increases strongly with increasing protein concentration above ~ 80g.L^{-1} due to jamming of the particles. The viscosity increase is stronger at lower temperatures caused by a strong decrease of the terminal relaxation time with increasing temperature. Addition of calcium ions introduces an attractive interaction that induces phase separation above a critical calcium concentration. Increasing the $CaCl_2$ concentration leads to an increase of the terminal relaxation time, but to a decrease of the high frequency elastic modulus. The effect of adding $CaCl_2$ is stronger at higher temperatures.

Introduction

Many food systems behave like soft solids, but still contain a large fraction of water. Such systems are often called gels and are easily recognized even though it is notoriously difficult to give an unambiguous definition of the concept gel. There are two fundamentally different ways to give aqueous food systems the texture of a soft solid.

The first way is to form a system spanning network of connected particles that can be proteins, polysaccharides or even small oil droplets. It does not matter whether the connections are covalent or physical bonds as long as they are sufficiently strong and long lived that the system does not flow or break easily.

The second method is to fill up the space with particles that do not bind to each other. In this case, the system stops flowing, because the particles are jammed by excluded volume interaction sometimes reinforced by electrostatic repulsion. Suspensions of non-interacting monodisperse hard spheres stop flowing close to the volume fraction of random close packing (ϕ=63%).[1] In food systems soft solids of this kind are usually formed by soft particles such as oil droplets or microgels. Because such particles can be deformed and compressed up to a certain extent, higher volume fractions are generally needed to obtain solid like behavior. In the case of microgels the particles may contain themselves a large fraction of water, which means that even if the particles are close packed the water content may still be high. Dense suspensions of monodisperse spheres form spontaneously a phase with crystalline order, but a small polydispersity or asymmetry is even to keep to system disordered. Therefore crystalline order is not found in food systems. Disordered solids are often called glasses and dense suspensions of hard spheres have extensively been studied as model systems for glasses in general.[2]

In the first case, the transition between a liquid-like and a solid-like behavior can be induced by increasing the number of strong bonds between particles leading to more and larger aggregates until at a critical amount one aggregate percolates the whole system. This process is often called the sol-gel transition and can be described by the percolation model.[3] In the second case, the liquid-solid transition can be induced by increasing the effective volume fraction of particles (ϕ_e). The theoretical description of this process, which is often called the glass transition, is not yet completely resolved even for the simplest case of hard spheres. [2] ϕ_e cannot only be increased by adding more particles, but one can also increase the effective volume of the particles *e.g.* by swelling or increasing the electrostatic repulsion.

The situation is more complex if there are attractive forces between the particles in addition to excluded volume interactions. The influence of short range attraction on the glass transition of hard spheres has been studied intensively and the term attractive glass has been coined to describe such systems. [4] Weak attraction was found to increase the volume fraction needed to induce the liquid-solid transition, but in the case of strong attraction it decreased. However, strong attraction leads to separation between a high density and a low density phase before the dynamics is arrested.[5] The domain where phase separation occurs reduced to lower concentrations and stronger attraction if the number of interaction sites on the particles is small.[6] In this case a percolating network can be formed with bonds that, although reversible, may be sufficiently strong and long-lived to cause gel-like behaviour.

Even if strong attraction will finally lead to phase separation, transient gel-like behavior may occur as initially the particles aggregate and form a system spanning network with long lived bonds that subsequently coarsens and evolves towards macroscopic phase separation.[7, 8] If the coarsening is slow compared to the time of observation such systems will behave as stable soft solids. If not, they may still behave as soft solids for some time until coarsening has progressed too far and the particles no longer percolate.

All these phenomena may be observed also in food systems. Here we investigate the liquid-solid transition for dense aqueous suspensions of caseinate. Caseins are by far the most abundant proteins in milk.[9] There are four major types of caseins each with a molar mass of around 20 $Kg.mol^{-1}$. In milk they are present in the form of spherical particles, so-called casein micelles, with a radius of about 100nm that are held together by calcium phosphate. The calcium phosphate can be removed by reducing the pH to the iso-electric point of the caseins where they precipitate. After washing, the caseins can be resolubilized by adding sodium hydroxide resulting in solutions of sodium caseinate (NaCas). [10]

When 0.1M or more NaCl is added, NaCas was found to form spontaneously small aggregates with a radius of about 11nm containing approximately 15 caseins, depending somewhat on the pH and the temperature .[11-13] Without added salt, similar aggregates are formed at higher concentrations.[14] The aggregation is most likely driven by hydrophobic interactions and it has been suggested that it is akin to micellisation of surfactants[15] as caseins contain relatively long chain sections with predominantly hydrophobic amino acids.[16] The rheology of dense NaCas suspensions has been studied in some detail.[14, 17-20] Above a concentration of about $C=80g.L^{-1}$ the viscosity increases rapidly with increasing concentration, which may be attributed to

jamming of the caseinate particles. The viscosity has a remarkably strong temperature dependence that is fully reversible. Pitkowski et al.[14] suggested that this temperature dependence could be explained by a weak decrease of the effective volume fraction with increasing temperature. In jammed suspensions a weak variation of ϕ_e causes a strong variation of the viscosity.

Added calcium ions bind specifically to caseins and can lead to aggregation of the caseinate particles. Alvarez et al.[21] reported changes in the intrinsic viscosity, fluorescence and circular dichroism spectra when $CaCl_2$ was added to dilute NaCas suspensions, which they attributed to the formation of increasingly dense aggregates. At higher $CaCl_2$ concentrations an increasing fraction of the proteins precipitates with increasing $CaCl_2$ concentration.[22, 23] The soluble fraction still consisted of small aggregates similar to those formed in the presence of NaCl.[22] Cuomo et al. 24 reported that for a given Ca^{2+} concentration the fraction of precipitated proteins increased with increasing temperature. The effect of adding calcium ions on the rheology of dense caseinate suspensions has not yet been investigated systematically.

Dense NaCas suspensions are a good example of a soft solid food system formed by jamming of dominantly repulsive soft particles. The aim of the present investigation was to study in more detail the rheology of dense NaCas suspension and the influence of attractive interaction of varying strength caused by adding different amounts of calcium ions. We will investigate the effects on the structure and the dynamic mechanical properties as a function of the protein concentration up to 300 $g.L^{-1}$ and the temperature (10-60°C) keeping the pH fixed at 6.7. It will be shown that addition of more than a critical amount of calcium ions drives phase separation. The results will be compared to those for suspensions of very similar caseinate particles obtained by addition of polyphosphates to native casein micelles that form soft solids by aggregating into a homogeneous percolating network.[25-27]

Materials and Methods

Materials. The sodium caseinate powder used for this study was provided by Armor protein (Saint-Brice, France). The water content was about 5wt%. The powders were dissolved in deionised water (Millipore) containing 3mM sodium azide as a bacteriostatic agent. The pH was adjusted to pH 6.7 by slow addition of 0.1M HCl or 1M NaOH under continuous stirring. Addition of $CaCl_2$ leads to a small decrease of the pH, which was compensated by addition of

NaOH. The casein concentration (C) was determined by measuring the nitrogen content (Kjeldahl) and the UV absorption at 280nm using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France). Consistent results were obtained using an extinction coefficient of 0.85 $L.g^{-1}.cm^{-1}$. The suspensions were homogenized by stirring at 80°C for about 30 min.

Rheology. Continuous and oscillatory shear measurements were made with a stress-imposed rheometer (TA Instruments Rheolyst AR2000) using a cone - plate geometry (40 mm, 0.58° or 20mm, 4.0°). For $C<165g.L^{-1}$ the measurements were done with another stress-imposed rheometer (MCR 301, Anton-Paar) using a Couette geometry (inner and out diameters: 26.6 and 28.9 mm). The temperature was controlled by a Peltier system and the geometry was covered with a mineral oil to prevent water evaporation.

Confocal Laser Scanning Microscopy. CLSM was used in the fluorescence mode. Observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany). A water immersion objective lens was used HCx PL APO 63x NA=1.2 with theoretical resolution 0.3µm in the x-y plane. Caseins were labelled with the fluorochrome rhodamine b isothiocyanate (Rho), by adding a small amount of a concentrated rhodamine solution to the casein solutions. Rho was excited using a helium–neon laser with a wavelength of 543 nm and the fluorescence was detected with a photomultiplier. Care was taken not to saturate the fluorescence signal and it was verified that the amplitude of the signal was proportional to the protein concentration.

Turbidity. The turbidity was determined as a function of the wavelength using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France). The path length through the cells was varied between 1 and 10 mm in order to obtain an optimum signal. The temperature was controlled with a thermostat bath.

Results and discussion

Dynamic mechanical properties of caseinate particle suspensions.

NaCas suspensions are transparent at low concentrations and translucent at higher concentrations. The latter is caused by a small weight fraction of particles with a radius of about 60nm (<5wt%). CLSM images of NaCas suspensions are homogeneous at all concentrations.

Small angle X-ray scattering of dense suspensions were compatible with that of close packed disordered particles with a diameter of about 20nm.[14]

Here we focus on the zero shear viscosity and the frequency dependence shear moduli in the limit of linear response, but it was shown elsewhere that concentrated suspensions of NaCas are shear thinning.[14, 19] Figure 1a shows the zero shear viscosity normalized by that of water (η_r) of NaCas suspensions as a function of the concentration for a range of temperatures. η_r rises very steeply with increasing concentration above about 80g.L^{-1}, but it does not diverge because the particles can interpenetrate.

The increase for C>120g.L^{-1} can be approximated by very steep power laws with exponents that decrease with decreasing temperature from about 15 at 10°C to 7 at 60°C. It was suggested in ref. 14 that the strong temperature dependence of η_r can to a large extent be explained by a small temperature dependence of the effective volume fraction. However, the more detailed investigation reported here shows that the data cannot be superimposed within the experimental error by simple horizontal shifts over the whole concentration range, see Figure 1b. When we superimpose the initial increase of the viscosity by shifting the concentrations, the data clearly deviate for C>120 g.L^{-1}. This means that in the jammed state the interaction between the particles depends on the temperature. We note that there is little effect of added NaCl (0-250mM) and the pH (5.4-6.7) on the viscosity[14], implying that electrostatic interactions are not very important.

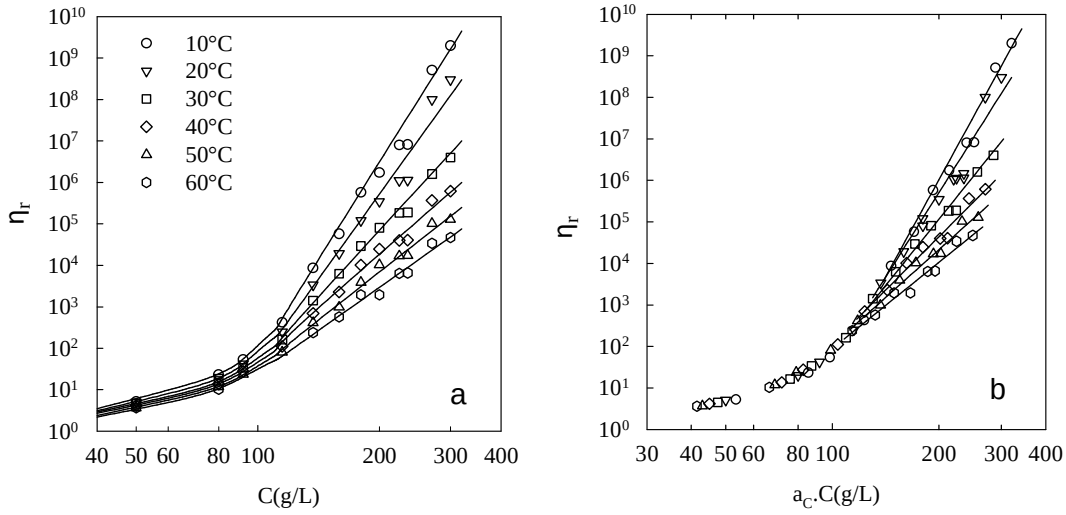

Figure 1a. Dependence of the relative zero shear viscosity as a function of the NaCas concentration for different temperatures indicated in the figure.

Figure 1b. Same data as in figure 1a after superimposition the data up to about of 120g.L^{-1} by horizontal shifts. The shift factors used are: 1.07, 1.0, 0.95, 0.9, 0.86 and 0.83 for 10, 20, 30, 40, 50 and 60°C, respectively.

The viscosity is determined by a combination of the high frequency elastic shear modulus (G_{el}) and the terminal relaxation time (τ): $\eta \propto G_{el}.\tau$. At higher protein concentrations these parameters could be determined separately by oscillatory shear measurements that showed an elastic response at high frequencies and a viscous response at low frequencies as reported earlier.[14, 19, 20] Oscillatory shear measurements made at different temperatures could be superimposed by frequency-temperature superposition. In this way master curves at T_{ref}=20°C were obtained at different concentrations, see Figure 2, that show that the stress relaxation is characterized by a broad relaxation time distribution. The terminal relaxation shifts rapidly to lower frequencies as the concentration is increased, while the elastic high frequency modulus increases only weakly. We note that the dynamic viscosity at low frequencies was equal to the low shear viscosity obtained from flow measurements.

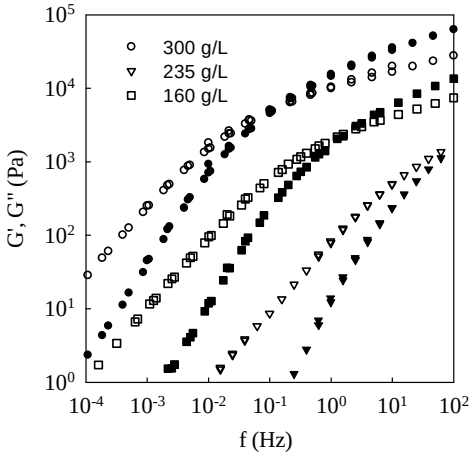

Figure 2. Master curves of the storage (closed symbols) and loss (open symbols) shear moduli obtained by temperature-frequency shifts at T_{ref}=20°C for different concentrations of NaCas indicated in the figure.

The terminal relaxation time was calculated from the frequency (f_c) where G' and G'' cross: $\tau=(2\pi f_c)^{-1}$. τ increased strongly with increasing concentration and decreasing temperature following a power law dependence, see Figure 3a. The elastic modulus was calculated as 10 times the value of G' at f_c. This choice is rather arbitrary, but the relative variation of G_{el} does not depend on this choice as the frequency dependence is almost independent of the temperature and the concentration. The concentration dependence of G_{el} is plotted in Figure 3b and shows that G_{el} is approximately proportional to C^2. It is clear that the increase of G_{el} with increasing concentration is much weaker than that of τ. G_{el} also increased systematically with increasing temperature, which taken on itself would lead to an increase of η_r with increasing temperature. However, this effect is overcompensated by the much stronger decrease of τ with increasing temperature. We may conclude that the main cause for the strong concentration and temperature dependence of η_r is the concentration dependence of τ.

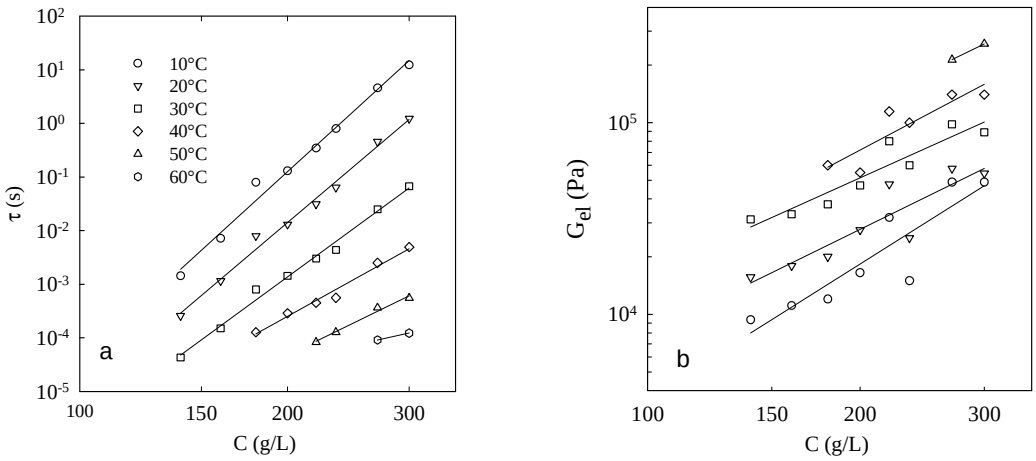

Figure 3. Dependence of the terminal relaxation time (a) and the high frequency elastic modulus (b) as a function of the NaCas concentration for different temperatures indicated in the figure. The solid lines were obtained from linear least squares fits.

On one hand, the increase of G_{el} with increasing temperature shows that the jammed particles are more difficult to deform at higher temperatures. On the other hand, the decrease of the relaxation time suggests that the particles can more easily escape from the cage formed by their neighbours. The increase of G_{el} with increasing temperature can be understood by the increase of the aggregation number of the particles and a small increase of their density that was observed in dilute solutions.[12] In the presence of 0.1M NaCl, both the hydrodynamic radius and the molar mass of the particles were found to increase with increasing temperature. As mentioned in the introduction we may suppose that the aggregates are formed by association of hydrophobic parts at the centre with a corona formed by the more hydrophilic chain sections. Within this scenario the increase of the aggregation number with increasing temperature is caused by an increase of the hydrophobic interactions. It is likely that the aggregation number and the density of the particles increase with increasing temperature also in the jammed state although the effect will be quantitatively different. Increased density renders the particles less deformable which explains qualitatively the temperature dependence G_{el}.

An increase of the density of the particles implies a decrease of their volume fraction, which explains why the suspensions jam at higher volume fraction when the temperature is increased. However, it does not explain the weaker dependence of the relaxation time on the concentration. On the contrary, one would expect that in the jammed state the relaxation time increases if the repulsion between the particles is stronger. For star polymers and polymeric micelles one observes a stronger concentration dependence of the viscosity when the aggregation number is

higher.[28, 29] As hydrophobic interactions become stronger with increasing temperature, it is very unlikely that the strong decrease of τ can be explained by an increase of the exchange rate of caseins between the caseinate particles. We speculate that in the case of caseinate particles weaker repulsion between the particles with increasing temperature leads to stronger interpenetration of the coronas and that this leads to stronger friction between the particles and thus a slower relaxation.

It is clear that even in the relatively simply situation of densely packed purely repulsive caseinate particles the dynamics cannot be explained solely in terms of escape from the cage formed by surrounding particles. Interactions on the molecular level between overlapping particles also need to be considered.

The influence of adding calcium ions on the structure.

Addition of $CaCl_2$ led to an increase of the turbidity above a critical amount (Cs*). At room temperature the increase was slow close to Cs*, but a stationary value was obtained rapidly at higher Cs and higher temperatures. Interestingly, we observed a strong reversible increase of the turbidity with increasing temperature, see Figure 4. Cs* increased somewhat with increasing protein concentration and decreasing temperature. At 20°C, Cs* was situated between 5 and 10mM at $C=20g.L^{-1}$ and between 10 and 15mM for $C=40g.L^{-1}$ and $C=80g.L^{-1}$. The system became turbid only when heated for $C=20g.L^{-1}$ at Cs=5mM and for $C=40g.L^{-1}$ at Cs=10mM.

At lower concentrations a fraction of the proteins precipitated slowly for Cs>Cs*. The strong increase of the viscosity inhibited sedimentation under gravity for $C>100g.L^{-1}$ at room temperature, but at elevated temperatures precipitation was also observed at higher concentrations. The fraction of soluble proteins (F) was quantified for $C=80g.L^{-1}$ and $C=20g.L^{-1}$ after incubating overnight at 20°C or at 40°C by determining the protein concentration in the supernatant after centrifugation (2h at $5.10^4 g$) at the incubation temperature. F decreased sharply for Cs>20mM at $C=80g.L^{-1}$ and for Cs>5mM at $C=20g.L^{-1}$. The drop was more important at the lower protein concentration and the higher temperature, see Figure 5. The reversibility was tested by incubating during overnight at 40°C and subsequently raising the temperature to 20°C. The same results were obtained as when the suspensions were incubated at 20°C, see Figure 5, demonstrating again that the temperature dependence was reversible.

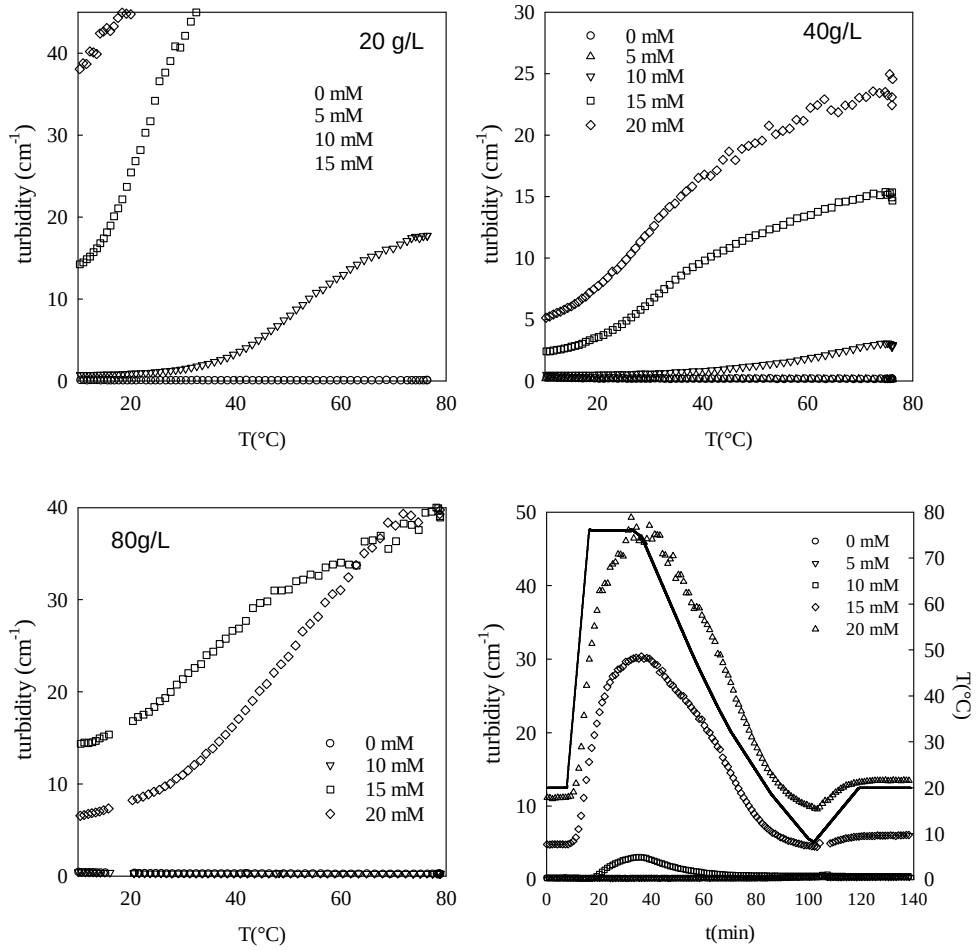

Figure 4. Temperature dependence of the turbidity at λ=800nm at different protein and $CaCl_2$ concentrations indicated in the figure. The reversibility is illustrated for C=40g.L^{-1} in the lower right panel that shows the turbidity as a function of the time during a heating and a cooling ramp. The sample temperature is indicated by the solid line.

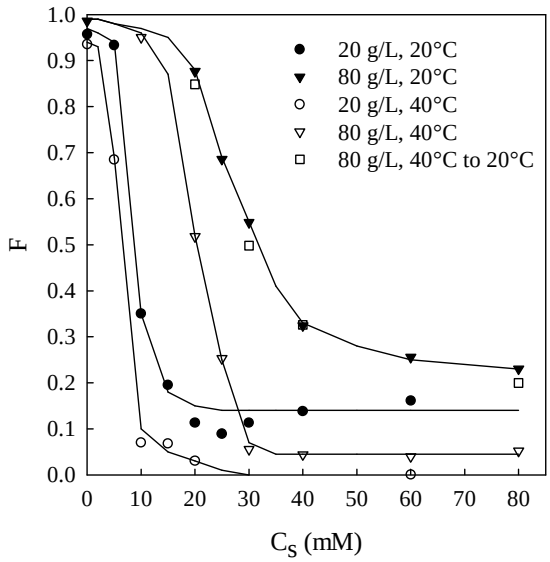

Figure 5. Fraction of soluble protein as a function of the $CaCl_2$ concentration at two protein concentrations and two temperatures. The effect of incubating at 40°C and subsequently cooling at 20°C is also shown.

The increase of the turbidity is caused by the formation of dense domains of proteins that at higher $CaCl_2$ concentrations become sufficiently large to be visible in the CLSM images, see Figure 6. Between 80 and 200g.L^{-1} protein the domains were visible when 30mM or more $CaCl_2$ was added, while at Cs=20M or lower the images were homogeneous. For C=235g.L^{-1} the images showed dense domains for Cs=40mM and higher and were homogeneous for Cs=30mM and lower. At lower protein and $CaCl_2$ concentrations small spherical domains were formed (see Figure 6a) that merged and took up a larger volume fraction at higher concentrations. If the protein and $CaCl_2$ concentrations were high enough the dense phase was continuous (see Figure 6d). By comparing the fluorescence intensity of the domains with the average intensity, we can estimate that the protein concentration within the domains is 250-300 g.L^{-1}. Suspensions containing 300 g.L^{-1} caseinate remained homogeneous after addition of $CaCl_2$, because in this case the dense phase filled up the whole space.

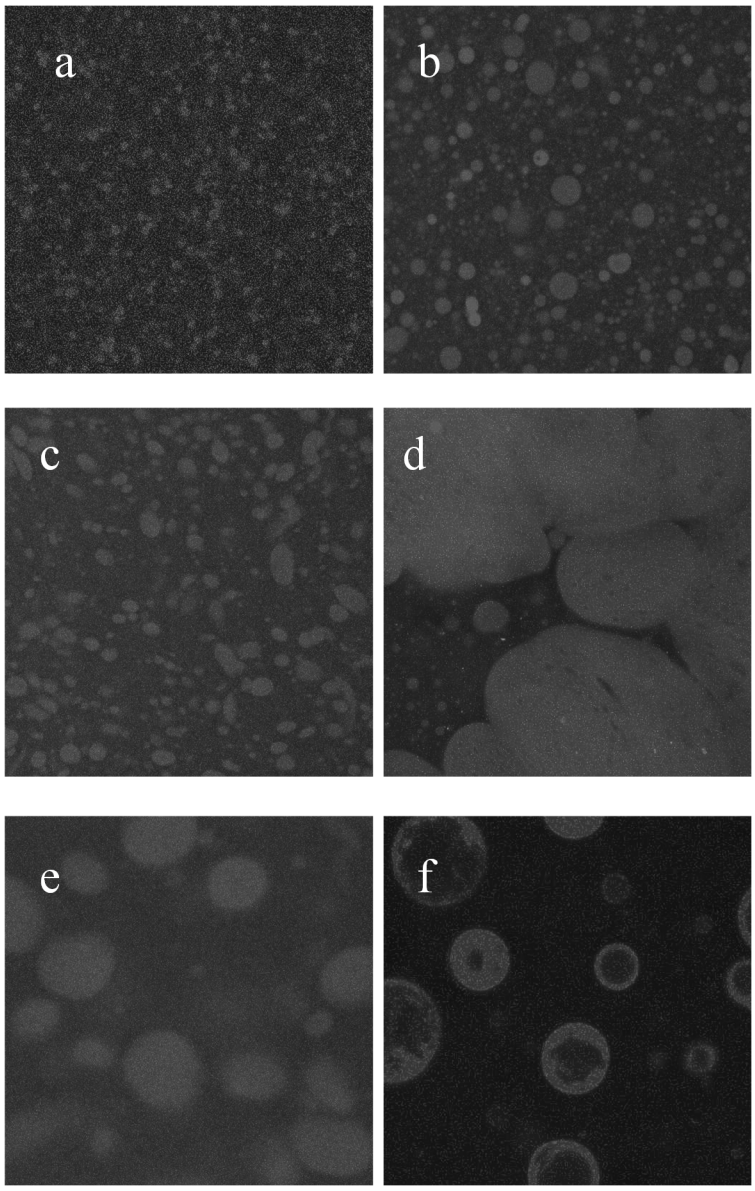

Figure 6. CLSM images of NaCas suspensions at different protein and $CaCl_2$ concentrations. a) C=80g.L^{-1},Cs=30mM; b) C=140g.L^{-1}, Cs=30mM; c) C=200g.L^{-1}, Cs=30mM; d) C=200g.L^{-1}, Cs=80mM; e) C=200g.L^{-1}, Cs=30mM; f) C=80g.L^{-1} Cs=12mM obtained by dilution with pure water from C=200g.L^{-1}, Cs=30mM. Images a-d represent 150x150µm and images e and f represent 75x75µm. For clarity, in all images the contrast between the phases has been artificially increased.

We investigated the reversibility of the phase separation by diluting a phase separated system at C=200g.L^{-1} and Cs=30mM (Figure 6e) with pure water to give C=80g.L^{-1} and Cs=12mM (Figure 6f). The solutions directly prepared at the latter conditions were homogeneous. We observed that after dilution most of the proteins in the dense domains redispersed, but the outer shell of the domains remained. In some cases we also observed an internal structure that looked like connected shells of several smaller domains that had merged. This observation shows that

strong bonds are formed specifically at the domain surfaces that do not break spontaneously when the concentration of calcium ions is reduced below the critical value.

The results discussed above together with those reported in the literature lead to the following overall picture of the effect of adding calcium ions on the structure of NaCas suspensions. Calcium ions bind specifically to caseins which induces an attraction between the caseinate particles that increases with increasing $CaCl_2$ concentration and temperature. Above a critical calcium concentration the system phase separates. The protein concentration in the dense phase is about 250g.L^{-1} and its volume fraction increases with increasing protein and calcium concentration. As a consequence, the density of the continuous phase decreases with increasing calcium concentration. When the volume fraction of the dense phase is not too high it is present in the form of spherical particles of a few microns. At the surface of the dense domains stable bonds are formed that resist to dilution in pure water.

The influence of adding calcium ions on the dynamic mechanical properties.

The effect of adding $CaCl_2$ on the visco-elastic properties of dense NaCas suspensions was studied in detail for selected concentrations between 80 and 300 g.L^{-1}. All measurements were done starting at 10°C and progressively increasing the temperature. The stability of the suspensions was tested by repeating the measurement at 20°C at the end of the experiment.

Figure 7 shows the dependence of the viscosity on the $CaCl_2$ concentration at different temperatures. For comparison we also show the corresponding values of the ratio (R) between the molar concentration of $CaCl_2$ and caseinate. At C=80g.L^{-1} addition of calcium ions caused a weak decrease of the viscosity. With increasing temperature the decrease became more important and started at lower calcium concentrations. At higher Cs, precipitation of proteins occurred rapidly especially at higher temperatures so that the range of Cs that could be investigated at this concentration was limited.

At C=140g.L^{-1} and 165g.L^{-1}we found a weak increase followed by a strong drop at higher Cs. In a rare investigation of the effect of adding $CaCl_2$ on the viscosity of NaCas suspensions Carr et al.[30] also reported a weak maximum for a NaCas suspension at 14 wt%. At C=200g.L^{-1} the viscosity was relatively insensitive to the addition of $CaCl_2$ except at higher temperatures where η_r decreased at larger Cs. Finally, at C=235g.L^{-1} and 300g.L^{-1} the viscosity increased initially with increasing Cs and then remained approximately constant at all temperatures. The suspensions with C≤165g.L^{-1}were not stable at higher temperatures when R was larger than

about 5, i.e. when the viscosity dropped sharply. This instability showed itself either by visible precipitation or by a lower viscosity at 20°C after the experiments at higher temperatures.

As was mentioned above, the dependence of the viscosity on Cs is caused by the combined effects on G_{el} and τ that could be studied separately for $C \geq 165 g.L^{-1}$. Again master curves of the frequency dependent shear moduli could be formed by frequency-temperature superposition. The shape of the master curves did not change significantly with the addition of $CaCl_2$, but the values of τ and G_{el} depended on Cs. The concentration dependence of τ and G_{el} is shown in Figure 8. We note that the spread of the data is much more important than in the absence of calcium ions. It is most likely caused by the heterogeneous structure seen in the CLSM images. The effect of phase separation on the structure may vary between different preparations and this will influence the visco-elastic properties. The absence of structural heterogeneity explains why the results at $C=300 g.L^{-1}$ are less scattered.

At all concentrations except at $C=300 g.L^{-1}$, G_{el} decreased with increasing $CaCl_2$ concentration. The decrease was stronger at higher temperatures and could even lead to an inversion of the temperature dependence from increasing with increasing temperature to decreasing with increasing temperature. At higher $CaCl_2$ concentrations and temperatures, the drop of the shear modulus became so strong that it was no longer possible to make useful oscillatory shear measurements.

At $C=165 g.L^{-1}$ the terminal relaxation time shows a clear maximum for all temperatures at about 50mM $CaCl_2$. Above this concentration the relaxation time dropped sharply. At higher protein concentrations adding $CaCl_2$ led to an increase of the relaxation time.

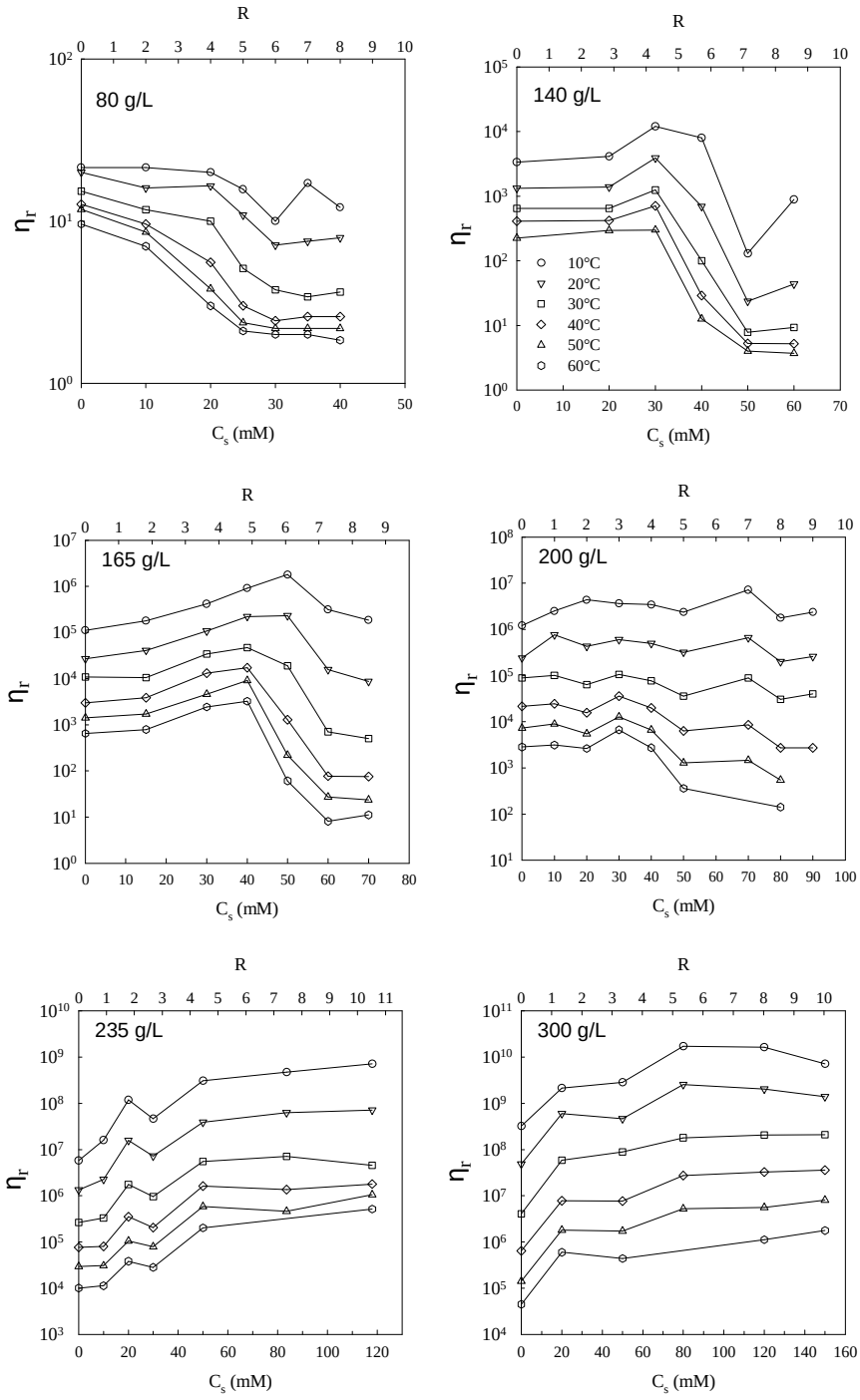

Figure 7. Dependence of the zero shear viscosity on the $CaCl_2$ concentration at different temperatures and different caseinate concentrations indicated in the figure. The top horizontal axis shows the corresponding values of the ratio (R) between the molar concentration of $CaCl_2$ and caseinate.

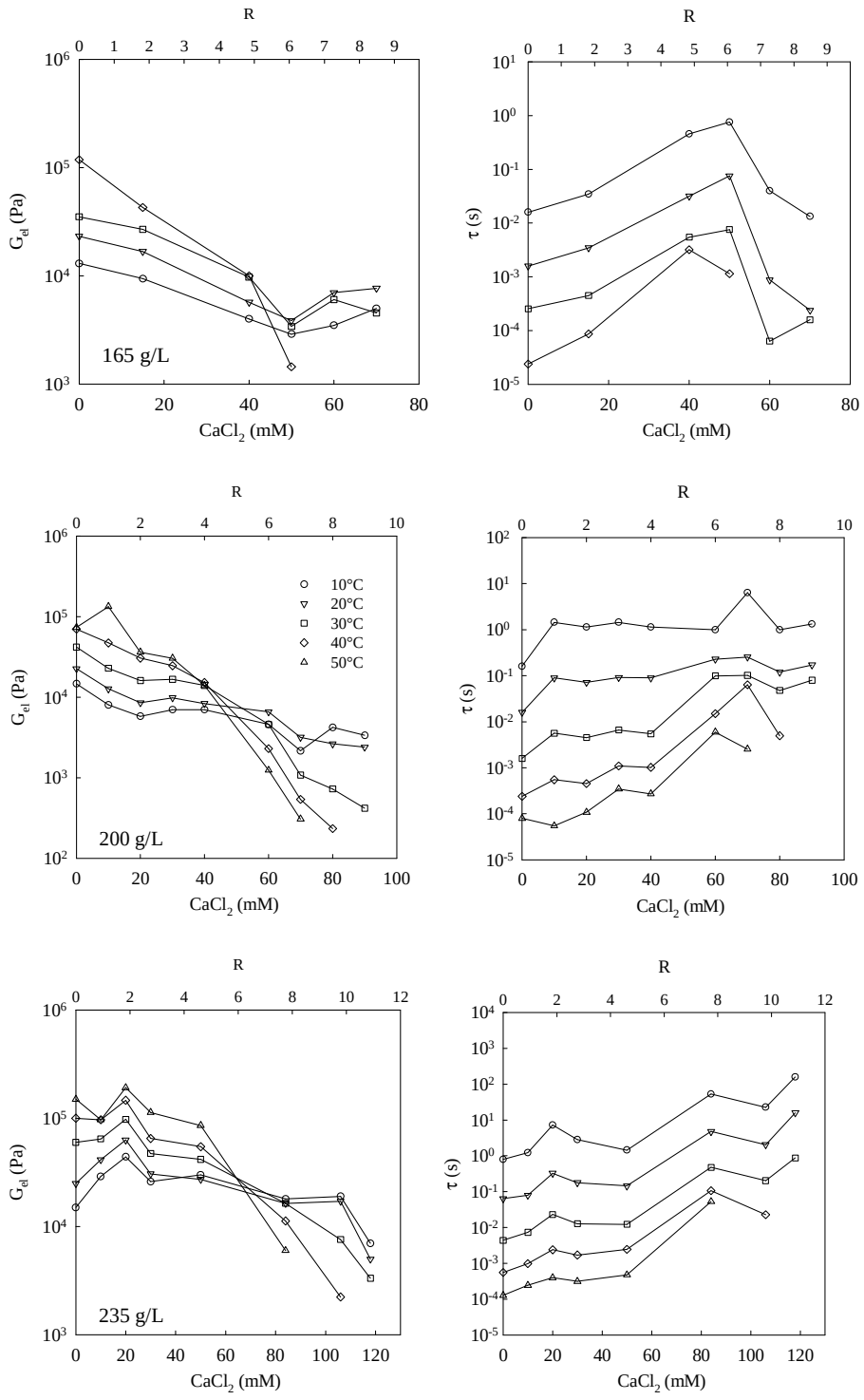

Figure 8. Dependence of the terminal relaxation time and the high frequency elastic modulus on the $CaCl_2$ concentration at different temperatures and caseinate concentrations indicated in the figure. The top horizontal axis shows the corresponding values of the ratio (R) between the molar concentration of $CaCl_2$ and caseinate.

As discussed above, adding calcium ions leads to an attractive interaction between the particles. Such an interaction is not expected to modify G_{el}, but increases the friction between the jammed particles and thus the terminal relaxation time. This may account for the behavior at 300g.L^{-1}. At lower protein concentrations we also need to consider the effect of phase separation which leads to a less dense continuous phase interspersed with dense domains. A decrease of the concentration of the continuous phase will lead to a decrease of G_{el}. A stronger phase separation at higher temperatures explains the stronger decrease of G_{el}.

Phase separation would also lead to a decrease of τ in the absence any effect of attractive interaction. The net effect of calcium ions on the relaxation time is thus determined by a balance between a reduction of the caseinate particle concentration in the continuous phase and an increase of the attractive interaction in this phase. At higher protein concentrations the volume fraction of the dense phase becomes large and may percolate through the system. Clearly, in this case the structure of the dense phase will also become important for its contribution to G_{el}.

Comparison with networks of caseinate particles.

When polyphosphate is added to an aqueous solution of native casein micelles, the latter disintegrate by chelation of the calcium inside the casein micelles. As a consequence, caseinate particles are formed with almost the same size as the sodium caseinate particles.[25] With increasing concentration the viscosity rises steeply similarly to NaCas. Also for this system the viscosity of dense suspensions decreases strongly with increasing temperature. An essential difference with the suspensions studied here, however, is that the particles irreversibly aggregate and form self-supporting gels at concentrations as low as 40 g.L^{-1}. The aggregation is very slow at room temperature, but the rate increases with increasing temperature. Clearly, the addition of polyphosphate together with the colloidal calcium phosphate causes the formation of strong bonds between the particles by a mechanism that has not yet been elucidated. Importantly, no phase separation was observed, though at low concentration the network was too weak to support its own weight leading to precipitation of large flocs. Dynamic mechanical measurements showed that the gelation process occurs through percolation of bound caseinate particles.[27] No relaxation was observed showing that the bonds were irreversible, contrary to the bonds formed by adding $CaCl_2$ to NaCas. This explains why in this system no phase separation occurred as densification is inhibited by the elasticity of the network. The elastic modulus increased with the square of the protein concentrations between 40–200 g.L^{-1}, similarly to G_{el} for NaCas suspensions at C > 140g.L^{-1}, but the absolute values

were somewhat smaller. Interestingly, no significant effect of the temperature on G_{el} was observed. This indicates that the elasticity for this system is determined by the connectivity of the network and not by the repulsion between jammed particles even at higher concentrations, where the connected particles are also clearly jammed.

Conclusion

Jamming, phase separation and network formation of the same caseinate particles can be induced in aqueous suspension by subtle variations of the mineral composition. Sodium caseinate forms small particles with a radius of about 10 nm in aqueous solution by association of hydrophobic chain sections. Above a concentration of about 80g.L^{-1} the particles jam causing a sharp increase of the viscosity. In the jammed state the viscosity increases following a power law with an exponent that decreases with increasing temperature.

For C > 140g.L^{-1} the visco-elastic properties of the suspensions can be characterized by oscillatory shear measurements. Master curves of the frequency dependence can be obtained by temperature–frequency superposition. They show a mainly elastic response at high frequencies and a relaxation characterized by a broad relaxation time distribution. The elastic modulus increases with increasing protein concentration and temperature. The latter can be understood by an increase of the aggregation number and the density of the caseinate particles with increasing temperature. The terminal relaxation time increases very strongly with increasing concentration and decreasing temperature and is the dominant cause of the variation of the viscosity. The dynamics of the jammed caseinate particles cannot be explained solely in terms of the escape of soft particles from the cage formed by neighbouring particles. Possibly, the increase of the relaxation time upon lowering the temperature is caused by increased interpenetration of the particles and thus stronger friction. Alternatively, it could be caused by hydrogen bond formation between particles. Addition of calcium ions causes an attractive interaction between the caseinate particles leading to phase separation above a critical concentration. Spherical dense protein domains of a few microns in diameter are formed that merge at higher protein concentrations. The volume fraction of the dense phase increases with increasing protein concentration until C= 300g.L^{-1} and higher, the dense phase occupies the whole volume. A small fraction of the particles in the dense domains form strong bonds that can withstand dilution in pure water. The addition of calcium ions causes a reduction of G_{el} which is more pronounced at higher temperatures. The decrease of G_{el} is probably caused by

the decrease of the protein concentration in the continuous phase when dense protein domains are formed. At higher protein and calcium concentrations the dense phase may percolate the system and contribute significantly to G_{el}, which implies that the structure of the dense phase also becomes an important parameter. At C = 300g.L^{-1} the effect of adding calcium ions is very small because the system remains homogeneous. The attraction between the particles caused by addition of calcium ions leads to an increase of the relaxation time. However, this effect is off-set by the reduction of the density of the continuous phase in more dilute solutions leading to a decrease in s at high $CaCl_2$ concentrations. Clearly, the effect of adding calcium ions on the dynamics of dense caseinate particle suspensions is determined by a complex interplay of increased attractive interaction and formation of a heterogeneous structure.

References

1. W. B. Russel, D. A. Saville and W. R. Schowalter, *Colloidal dispersions*, Cambridge Univ Pr, 1992.

2. F. Sciortino and P. Tartaglia, *Advances in Physics*, 2005, 54, 471-524.

3. D. Stauffer and A. Aharony, *Introduction to percolation theory*, Taylor & Francis, London, 1992.

4. K. A. Dawson, *Current Opinion in Colloid and Interface Science*, 2002, 7, 218-227.

5. G. Foffi, C. De Michele, F. Sciortino and P. Tartaglia, *Physical Review Letters*, 2005, 94, 078301-078301-078304.

6. F. Sciortino and E. Zaccarelli, *Current Opinion in Solid State and Materials Science*, 2011, 15, 246–253.

7. S. Babu, J.-C. Gimel and T. Nicolai, *Journal of Chemical Physics*, 2006, 125, 184512-184511-184510.

8. W. C. K. Poon, L. Starrs, S. P. Meeker, A. Moussaïd, R. M. L. Evans, P. N. Pusey and M. M. Robins, *Faraday Discussions*, 1999, 112, 143-154.

9. P. F. Fox, *Advanced Dairy Chemistry vol.1*: Proteins 3rd editions, ed. K. A. P.F. Fox and P.L.H. McSweeney, 2003, pp. 427-435.

10. C. R. Southward, *Developement in Dairy Chemistry-4*, ed. F. P. Fox, Elsiever Applied Science, London, 1989, pp. 173-244.

11. B. Chu, Z. Zhou, G. Wu and H. M. J. Farrell, *Journal of Colloid and Interface Science*, 1995, 170, 102-112.

12. A. HadjSadok, A. Pitkowski, L. Benyahia, T. Nicolai and N. Moulai-Mostefa, *Food Hydrocolloids*, 2008, 22: 1460-1466.

13. J. A. Lucey, M. Srinivasan, H. Singh and P. A. Munro, *Journal of Food Chemistry*, 2000, 48, 1610-1616.

14. A. Pitkowski, D. Durand and T. Nicolai, *J. Coll. Interf. Sci*. 2008, 326, 96-102.

15. C. G. de Kruif and V. Y. Grinberg, *Colloids and Surfaces. A:Physicochem. Eng. Aspects*, 2002, 210, 183-190.

16. D. S. Horne, *Current Opinion in Colloid and Interface Science*, 2002, 7, 456-461.

17. D. Farrer and A. Lips, *International Dairy Journal*, 1999, 9, 281-286.

18. J. Fichtali, F. R. van de Voort and G. J. Doyon, *Journal of Food Engineering*, 1993, 19, 203-2011.

19. S. M. Loveday, M. A. Rao, L. K. Creamer and H. Singh, *Journal of Food Science*, 2010, 75, N30-N35.

20. A. Bouchoux, P. E. Cayemitte, J. Jardin, G. Gasan-Guiziou and B. Cabane, *Biophysical journal*, 2009, 96, 693-706.

21. E. M. Alvarez, P. H. Risso, M. A. M. Canales, M. S. Pires and C. A. Gatti, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2008, 327, 51-56.

22. A. Pitkowski, T. Nicolai and D. Durand, *Food Hydrocolloids*, 2009, 4, 1164-1168.

23. C. A. Zittle and E. S. Dellamonica, *Archives of Biochemistry and Biophysics*, 1958, 76, 342-353.

24. F. Cuomo, A. Ceglie and F. Lopez, *Food Chemistry*, 2011.

25. M. Panouillé, L. Benyahia, D. Durand and T. Nicolai, *J. Coll. Int. Sci.*, 2005, 287, 468.

26. M. Panouillé, D. Durand, T. Nicolai, N. Boisset and E. Larquet, *J. Coll. Int. Sci.*, 2005, 287, 85.

27. A. Pitkowski, T. Nicolai and D. Durand, *J. Rheology*, 2007, 52, 971.

28. J. Roovers, *Macromolecules*, 1994, 27, 5359-5364.

29. D. Vlassopoulos, G. Fytas, S. Pispas and N. Hadjichristidis, *Physica B: Condensed Matter*, 2001, 296, 184-189.

30. A. J. Carr, P. A. Munro and O. H. Campanella, *Journal of Dairy Research*, 2002, 12, 487-492.

Chapter 4.2. Comparative study of the rheology and the structure of sodium and calcium caseinate solutions.

Published in *International Dairy Journal (2013)* 31 (2013) 100-106.

Peggy Thomar, Taco Nicolai, Lazhar Benyahia, Dominique Durand.

LUNAM Université du Maine, IMMM UMR-CNRS, Polymères, Colloïdes et Interfaces, 72085 le Mans Cedex 9, France

Email : Taco.Nicolai@univ-lemans.fr

Abstract

The viscosity and the frequency dependent shear moduli of dense calcium caseinate (CaCN) and sodium caseinate (NaCN) suspensions were investigated as a function of concentration (50-300g.L^{-1}) and temperature (10-50°C) at pH 6.7. Both systems form visco-elastic suspensions with a viscosity that increases strongly with increasing concentration and decreasing temperature. An important effect of the counter-ion on the visco-elastic properties was observed that is attributed to the specific binding of calcium, which reduces the electrostatic repulsion and induces a net attractive interaction between caseins. The latter may lead to micro-phase separation as was observed by confocal laser scanning microscopy. Mixtures of NaCN and CaCN were significantly different from pure NaCN if they contained more than 50% CaCN. The equivalence between NaCN with added $CaCl_2$ and CaCN is discussed.

1. Introduction

In milk, the caseins are assembled into large aggregates called casein micelles, which are held together by colloidal calcium phosphate (CCP) (Horne, 2006). The CCP can be removed by lowering the pH to the iso-electric point, which leads to precipitation of the casein, and washing. Subsequently, the casein can be resuspended by increasing the pH with NaOH or $Ca(OH)_2$, resulting in aqueous solutions of sodium caseinate (NaCN) or calcium caseinate (CaCN), respectively. Both NaCN and CaCN are produced industrially on a large scale and have found applications in a range of food products (Mulvihill & Fox, 1989; Southward, 1989). In aqueous solution, NaCN forms small aggregates with a radius of about 11 nm containing about 15 casein molecules, depending on temperature, pH and the ionic strength (Chu, Zhou, Wu, & Farrell, 1995; HadjSadok, Pitkowski, Benyahia, Nicolai, & Moulai-Mostefa, 2008; Lucey, Srinivasan, Singh, & Munro, 2000). The rheology of NaCN suspensions has already been studied in considerable detail (Bouchoux et al., 2009; Farrer & Lips, 1999; Fichtali, Van de Voort, & Doyon, 1993; Loveday, Rao, Creamer, & Singh, 2010; Pitkowski, Durand, & Nicolai, 2008; Thomar, Durand, Benyahia, & Nicolai, 2012). Dense suspensions of NaCN ($C > 100 g.L^{-1}$) are visco-elastic and their viscosity increases strongly with increasing concentration and decreasing temperature. The increase of the viscosity was explained by jamming of the close packed small casein particles (Pitkowski et al., 2008). The temperature dependence could in part be explained by a decrease of the effective volume fraction with increasing the temperature and in part by a decrease of the friction between the particles (Thomar et al., 2012). It is well known that κ-casein is insensitive to calcium ions, whereas the other caseins specifically bind $Ca^{2+,}$ which reduces electrostatic repulsion and induces aggregation (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981; Swaisgood, 1993; Zittle & Dellamonica, 1958). The effect of adding $CaCl_2$ on the aggregation of α_{s1}-casein (Dalgleish & Parker,1980; Dalgleish, Paterson, & Horne, 1981; Farrell, Kumosinski, Pulaski, & Thompson, 1988; Horne, 1983) and β-casein (Farrell et al., 1988; Guo, Campbell, Chen, Lenhoff, & Velev, 2003; Parker & Dalgleish, 1981) in dilute solution has been investigated in some detail. In NaCN suspensions, addition of $CaCl_2$ leads to the formation of micron sized dense protein domains that agglomerate and precipitate in more dilute suspensions (Cuomo, Ceglie, & Lopez, 2011; Lopez, Cuomo, Lo Nostro, & Ceglie, 2012). Consequently, adding calcium ions strongly decreases the solubility of caseinate (Pitkowski, Nicolai, & Durand, 2009; Thomar et al., 2012). However, the soluble fraction still consists of small aggregates similar to those formed in the presence of NaCl (Pitkowski et al., 2009). The formation of dense protein domains in the presence of Ca^{2+} increases the turbidity

and influences the rheological properties of dense caseinate suspensions (Thomar et al., 2012). Studies of CaCN have focused in most cases on its emulsifying properties (Sosa-Herrera, Lozano, Ponce de León, & Martínez-Padilla, 2012; Srinivasan, Singh, & Munro, 1999; Ye, Srinivasan, & Singh, 2000) and its capacity for film (Monedero, Fabra, Talens, & Chiralt, 2010) and fibre formation (Grabowska, van der Goot, & Boom, 2012; Manski, van der Goot, & Boom, 2007). As far as we are aware, the morphology and the rheology of dense CaCN suspensions have not yet been investigated in detail. The objective of the present study was to characterise the microscopic structure, the frequency dependent shear moduli and the viscosity as a function of the casein concentration and the temperature. The results were compared with those obtained on NaCN. We have also studied mixtures of NaCN and CaCN, and discuss the difference between CaCN and NaCN with the equivalent amount of Ca^{2+} added in the form of $CaCl_2$.

2. Materials and methods

2.1. Materials. NaCN and CaCN powders used for this study were provided by Armor Protein (Saint-Brice, France). According to the manufacturer, the CaCN powder contained 1.3% (w/w) calcium and the NaCN powder contained 1.3% (w/w) sodium. In both cases, this amount corresponds approximately to the counterions and the amount of excess salt is very small. The water content was 5% (w/w) for both powders. The powders were dissolved in deionised water containing 3mM sodium azide as a bacteriostatic agent. The pH was adjusted to pH 6.7 by slow addition of 0.1M HCl or 1M NaOH under continuous stirring. The casein concentration (C) was determined by measuring the nitrogen content (Kjeldahl) and UV absorption at 280nm using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France). Consistent results were obtained using an extinction coefficient of 0.85 $L.g^{-1}.cm^{-1}$. The suspensions were prepared by stirring at 80°C for about 20 min.

2.2. Rheology. Continuous and oscillatory shear measurements were made with a stress-imposed rheometer (AR2000, TA Instruments, Guyancourt, France) using a cone-plate geometry (40 mm, 0.58° or 20 mm, 4.01°). For $C<165g.L^{-1}$ the measurements were performed with another stress-imposed rheometer (MCR 301, Anton-Paar, Graz, Austria) using a Couette geometry (inner and out diameters: 26.6 and 28.9 mm). The temperature was controlled by a Peltier system and the geometry was covered with a mineral oil to prevent water evaporation.

2.3. Confocal Laser Scanning Microscopy. Confocal Laser Scanning Microscopy (CLSM) was used in the fluorescence mode. Observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany). A water immersion objective lens was used HCx PL APO 63x NA= 1.2 with a theoretical resolution 0.3mm in the x-y plane. The caseins were labeled with the fluorochrome rhodamine b, by adding a small amount of a concentrated rhodamine solution to the solutions. The rhodamine was excited using a helium neon laser with wavelength 543nm and the fluorescence was detected with a photomultiplier. Care was taken not to saturate the fluorescence signal and it was verified that the amplitude of the signal was proportional to the protein concentration.

3. Results and discussion

3.1. Structure of calcium caseinate suspensions

For the CaCN sample used here, the ratio (R) between the molar concentrations of calcium and caseinate is equal to 7. The study was performed at pH 6.7 where the average charge density of caseinate is close to -14 (Swaisgood, 1993). Consequently, almost all calcium ions are counterions. Aqueous solutions of CaCN were turbid at all concentrations investigated (10-300g.L^{-1}) whereas NaCN solutions were transparent at low concentrations and translucent at higher concentrations. CLSM images of suspensions of CaCN show spherical dense domains of proteins with diameters of several microns (Figure 1).

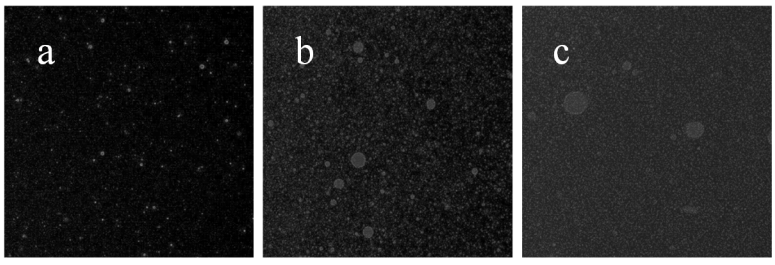

Figure 1. CLSM images of suspensions of calcium caseinate for different protein concentrations: a, C= 10 g.L^{-1}; b, C= 130g.L^{-1}; c, C = 235 g.L^{-1}. Images represent 150x150mm. For clarity, in all images the contrast between the phases has been artificially increased.

This suggests that the presence of calcium counterions is sufficient to induce micro-phase separation at concentrations as low as 10g.L^{-1}. The protein concentration of the dense phase

was about 250g.L^{-1} similar to that reported for the protein domains that formed when $CaCl_2$ was added to NaCN (Thomar et al., 2012). The dense protein domains were precipitated by centrifugation (2 h at 50,000*g*) after incubating overnight between 10 and 40°C. The fraction of soluble proteins (F) remaining in the supernatant was determined as a function of the total protein concentration (C =10-80g.L^{-1}; Figure 2).

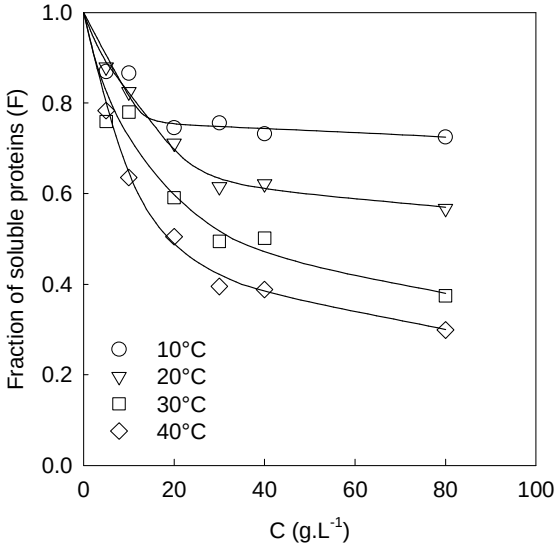

Figure 2. Fraction of soluble proteins (F) in the supernatant after centrifugation of calcium caseinate suspensions as a function of the protein concentration for different temperatures indicated in the figure.

F decreased sharply with increasing CaCN concentration up to about 30g.L^{-1} and weakly at higher concentrations. The decrease was more pronounced at high temperatures suggesting that the calcium induced interaction increased with increasing temperature. A decrease of the solubility with increasing temperature was also reported for NaCN with added $CaCl_2$ (Thomar et al., 2012). It has been reported earlier for dilute solutions of pure α_{s1}- and β-casein that Ca^{2+} binds more strongly at higher temperatures leading to a lower critical $CaCl_2$ concentration for precipitation (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981).

3.2. Rheology of calcium caseinate suspensions

As mentioned in the introduction, the rheology of NaCN solutions has already been investigated in some detail. Here we show results obtained for CaCN. The zero shear viscosity and the frequency dependent shear moduli were measured over a wide range of concentrations (50-300g.L^{-1}) and temperatures (10-50°C). It was found that the shear moduli of CaCN were not stable immediately after the set temperature was reached, but continued to increase (Figure 3). For the measurements shown here we equilibrated the samples at each temperature for 20 min. The results depend somewhat on the choice of the equilibration time, but the effect is relatively small compared with the large effects of varying the temperature and the protein concentration.

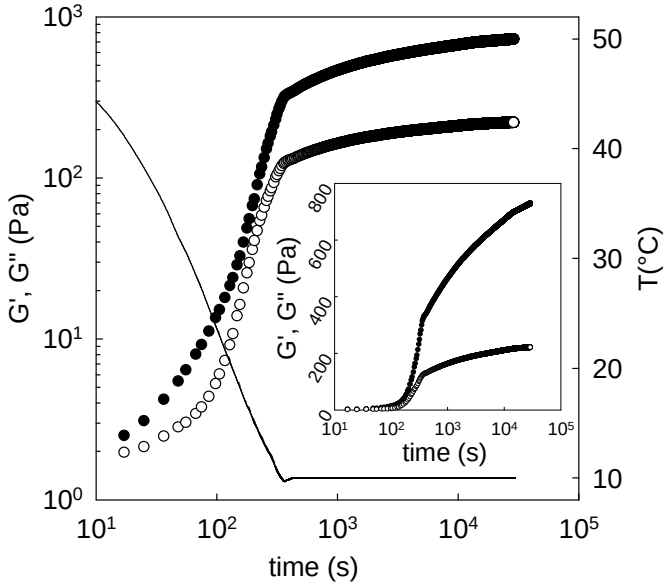

Figure 3. Log-log representation of the time dependence of the storage G' (filled symbols) and loss G" (open symbols) shear moduli at 1Hz during and after lowering the temperature from 50°C to 10°C for a suspension of calcium caseinate at C =180 g.L^{-1}. The log-lin representation (inset) shows that G' and G" continued to increase significantly after the set temperature was reached. The solid line indicates the temperature.

Figure 4a shows the frequency dependence of the shear moduli at different temperatures for a CaCN suspension at C =235g.L^{-1}. At 10 °C the system showed a weak frequency dependence of the moduli and G' was larger than G" over the whole frequency range investigated. At higher temperatures a cross-over between G' and G" appeared that shifted rapidly to higher frequencies with increasing temperature, while the elastic modulus decreased. A master curve could be obtained by frequency-temperature superposition of the data between 10°C and 40°C (Figure 4b). The master curve shows clearly that the dense CaCN suspension is visco-elastic

and characterised by a broad distribution of relaxation times. Similar behaviour has already been reported for dense NaCN suspensions with and without added $CaCl_2$ (Thomar et al., 2012).

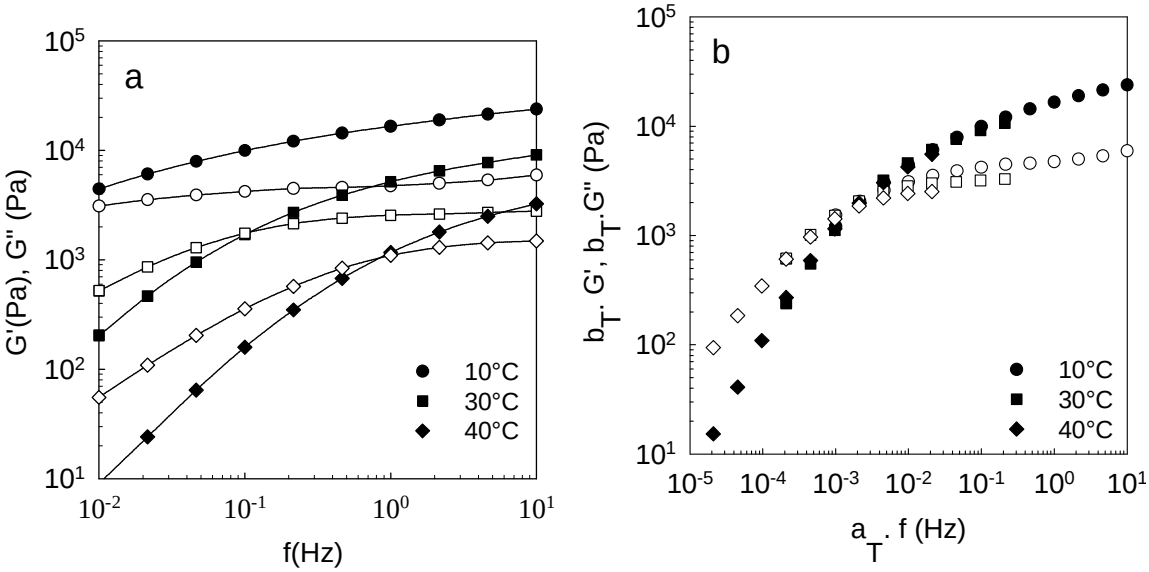

Figure 4. Frequency dependence of the storage G' (filled symbols) and loss G" (open symbols) shear moduli (panel a) at different temperatures for a suspension of calcium caseinate at C=235 g.L^{-1} and panel b, master curve of the data shown in panel a obtained by frequency-temperature superposition at a reference temperature of 10°C.

The zero-shear viscosity was derived from the oscillatory shear measurements at low frequencies. It was checked that the same values were obtained using flow measurements at low shear rates. Highly concentrated CaCN suspensions showed, similarly to NaCN, shear thinning behaviour (results not shown).

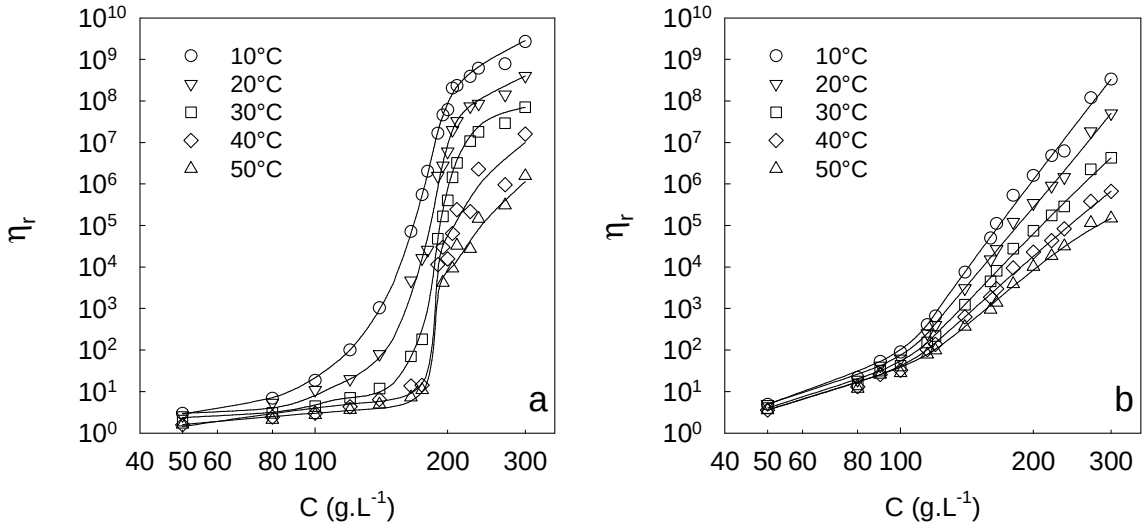

Figure 5. Relative zero shear viscosity (η_r) as a function of the protein concentration for calcium (a) and sodium (b) caseinate at different temperatures.

Figure 5a shows the zero shear viscosity normalised by that of water (η_r) of CaCN suspensions as a function of the protein concentration for a range of temperatures. For comparison, the results obtained on NaCN that were reported by Thomar et al. (2012) are shown in Figure 5b. In both cases, η_r increased strongly with increasing protein concentration and decreased with increasing temperature.

The strong increase of η_r with increasing concentration for C >80g.L^{-1} for NaCN can be understood in terms of jamming of soft caseinate particles. The presence of calcium leads to the formation of dense caseinate domains, which dramatically alters the visco-elastic properties of CaCN suspensions compared with NaCN suspensions. At low protein concentrations, the viscosity of CaCN is lower than that of NaCN due to the formation of the dense domains, which reduces the effective volume fraction of the continuous medium. At a critical concentration that depends on the temperature, η_r increases sharply for CaCN, because the dense domains percolate and form a system spanning network. The relatively weak increase of η_r at higher protein concentrations is caused by the increase of the volume fraction of the dense phase.

For both CaCN and NaCN, the decrease in η_r with increasing temperature can be partially explained by a decrease of the effective volume fraction of the small caseinate particles. However, for CaCN, the effect of increasing temperature on η_r is amplified at lower concentrations by the increased micro-phase separation. This caused the very low viscosity at higher temperatures for C < 190g.L^{-1}. At higher protein concentrations (C > 160g.L^{-1}) the

viscoelastic behaviour could be characterised by measurements of G' and G" as a function of the frequency, which allowed us to determine the terminal relaxation time (s) and the high frequency elastic modulus (G_e).

τ was calculated from the frequency (f_c) where G' and G" crossed: $\tau = 1/(2\pi f_c)$, and G_e was taken as 10 times the value of G' at f_c. The choice of the exact values of τ and G_e is necessarily somewhat arbitrary because the relaxation process is broad. However, the choice does not influence the concentration and temperature dependence as the relaxation time distribution is similar in all cases. The concentration dependence of τ at different temperatures is compared in Figure 6 for CaCN and NaCN.

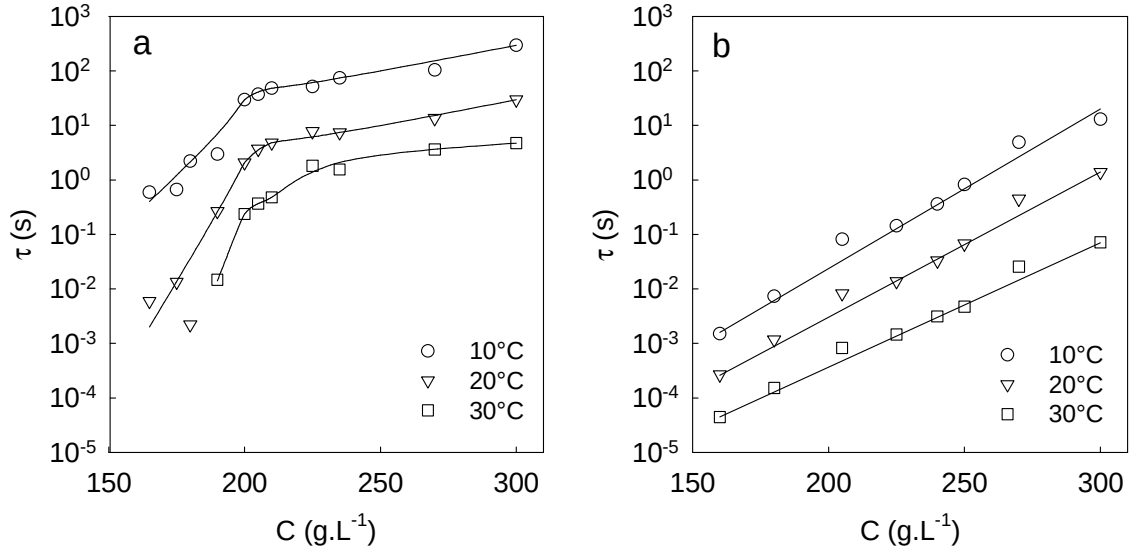

Figure 6. Dependence of the terminal relaxation time (τ) on protein concentration for calcium (a) and sodium (b) caseinates at different temperatures.

For NaCN, τ increased exponentially with increasing concentration over the whole range between 160 and 300g.L^{-1}, while for CaCN it increased initially very steeply, but only weakly for C > 200 g.L^{-1}. Increasing the temperature caused a systematic shift of τ to shorter times. At all concentrations, the absolutes values of τ were higher for CaCN than for NaCN. The concentration dependence of G_e is shown in Figure 7. Both for CaCN and NaCN the concentration dependence of G_e was relatively weak compared with that of τ, implying that the variation of η_r is mainly controlled by that of τ. As for τ, we observed for NaCN a continuous increase of G_e with increasing C over the whole range, while for CaCN we found an initial steep

increase followed by a very weak increase. G_e increased weakly with increasing temperature for NaCN, while it decreased weakly for CaCN.

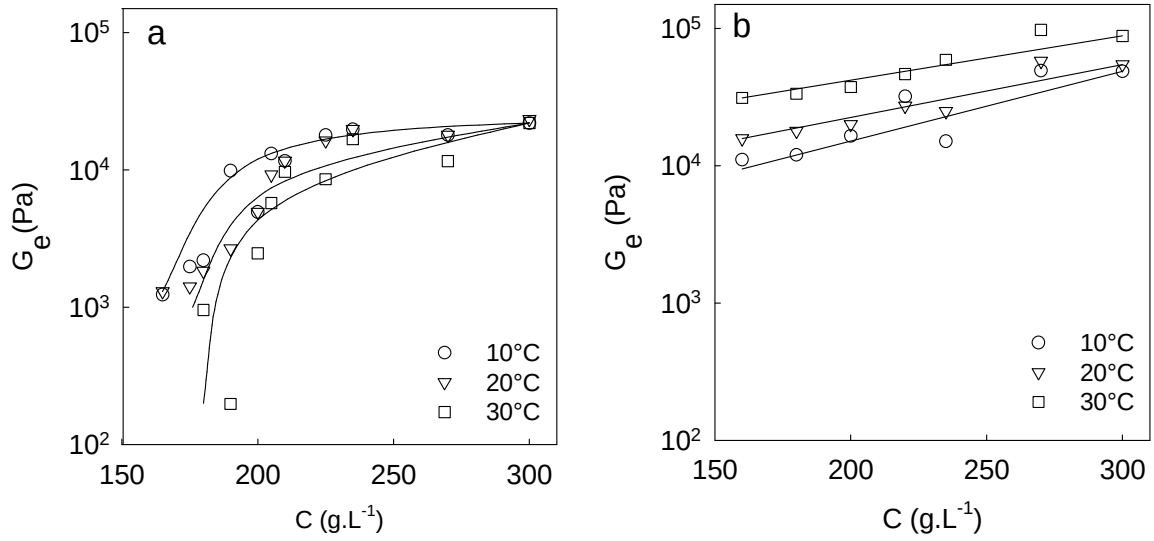

Figure 7. Elastic modulus (G_e) of calcium (a) and sodium (b) caseinates suspensions as a function of the protein concentration at different temperatures.

The sharp increase in G_e and τ for CaCN with increasing concentration and the corresponding sharp rise of η_r suggests that the visco-elastic suspension is formed by percolation of the dense protein domains. Once the domains form a system spanning network the stress needed to strain the system is determined by the domains and no longer by the suspension of small casein particles in which the domains are embedded. The network of dense domains is still tenuous at 160 g.L^{-1}, but quickly becomes stronger as more domains come in contact and are strained. This behaviour is very different from that of NaCN, which forms visco-elastic suspensions by jamming of the small casein particles. The latter are less dense and more deformable than the dense protein domains causing a more progressive increase of the viscosity starting at lower concentration. Note that at low temperatures, where micro-phase separation is less important, the effect of jamming of the small casein particles can still be observed for CaCN at lower concentrations. The different origin of the elasticity for NaCN and CaCN also explains the different temperature dependence of G_e. For CaCN, the sharp rise of G_e due to percolation of the dense domains started at slightly higher concentrations at higher temperatures. We speculate that this is caused by an increase of the density of the protein domains with increasing temperature so that they percolate at a higher concentration. For NaCN, G_e increased more progressively with increasing temperature. This is remarkable because τ decreased strongly

with increasing temperature. A possible explanation for this behaviour is that the small casein particles become less easy to deform with increasing temperature, causing the increase of G_e, but that the friction between the particles decreases causing a decrease of τ. The same phenomena were earlier reported for NaCN suspensions with added $CaCl_2$ and it was suggested that with increasing temperature the small particles become less entangled (Thomar et al., 2012).

3.3. Mixtures of sodium caseinate and calcium caseinate

We have investigated mixtures of NaCN and CaCN solutions with a range of protein concentrations between 120 and 235g.L^{-1}. At room temperature, the solutions became visually turbid when the fraction of CaCN was higher than 60%. CLSM showed an increasing amount of dense protein domains with increasing fraction of CaCN starting at 50% (R =3.5) (Figure 8). It appears that microphase separation occurs when more than 3 calcium ions are present per casein molecule. The fact that at lower fractions of CaCN the mixtures are homogeneous implies that the calcium ions redistribute among all caseins. The kinetics of this redistribution is relatively fast as turbid CaCN solutions become transparent during mixing with NaCN solutions.

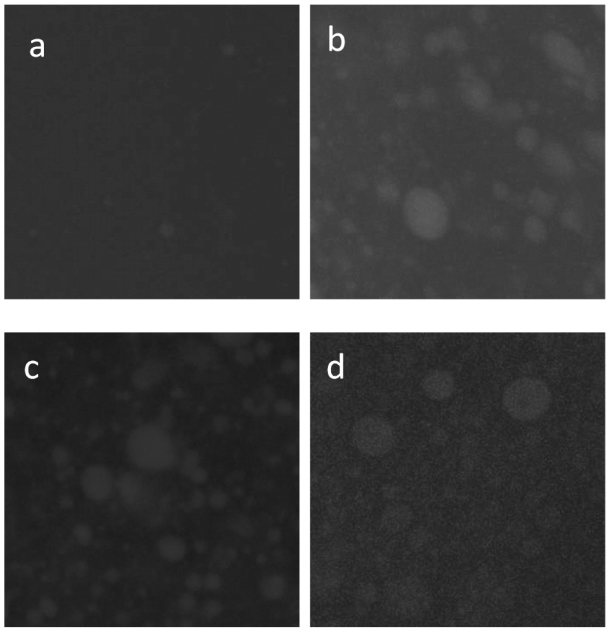

Figure 8. CLSM images of mixtures of sodium (NaCN) and calcium caseinates (CaCN) suspensions at C=215g.L^{-1} with different fractions of CaCN. a) CaCN 50% (R=3.5); b) CaCN 60% (R=4.2); c) CaCN 70% (R=4.9); d) CaCN 100% (R=7). Images represent 40x40µm. For clarity, the contrast of the images between the phases has been artificially increased.

If less than 60% CaCN was present in the mixtures, their viscosity was very close to that of pure NaCN. In Figure 9, the concentration dependence of η_r of two mixtures containing 60% and 85% CaCN is compared with that of pure NaCN and CaCN at 20°C. The behaviour is intermediate at low and high protein concentrations. However, in a narrow range between 150 and 190g.L^{-1} the viscosity appears to be slightly larger for the mixtures than for either of the pure systems. This is probably a consequence of two opposing effects: on one hand, when the content of CaCN is decreased, the characteristic sharp increase of the viscosity for pure CaCN shifts to lower protein concentrations. However, on the other hand, the rise becomes less pronounced. Both effects can be explained by a decrease of the density of the protein rich domains with decreasing CaCN content.

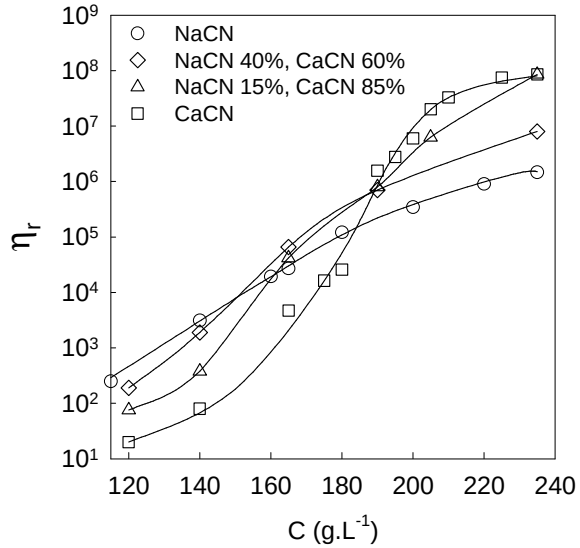

Figure 9. Relative zero shear viscosity (η_r) of different mixtures of sodium and calcium caseinates (R= 0; 4.2; 6; 7) as a function of the protein concentration at 20°C.

The present results are compatible to measurements of the viscosity as a function of the ratio NaCN/CaCN at C=140 g.L^{-1}, which show a small increase up to 60% CaCN and a sharp decrease at higher CaCN concentrations (Carr, Southward, & Creamer, 2003).

3.4. Comparison between calcium caseinate and sodium caseinate with $CaCl_2$

An important question is whether the behaviour of CaCN is the same as that of NaCN with added $CaCl_2$. Therefore we compared the concentration dependence of η_r for NaCN solutions with different amounts of added $CaCl_2$ with that of CaCN. If 8 Ca^{2+} ions were added per molecule the behaviour was very close to that of CaCN (Figure 10). This ratio is within the experimental error the same as that in the CaCN, which means that it is equivalent to use CaCN produced by neutralization with calcium hydroxide or calcium carbonate or NaCN with the same amount of Ca^{2+} added in the form of $CaCl_2$. The difference is that in the latter case the solutions also contain sodium ions (up to 170mM for C=300 g.L^{-1}). However, we observed only a small effect of adding 200mM NaCl on the viscosity of CaCN.

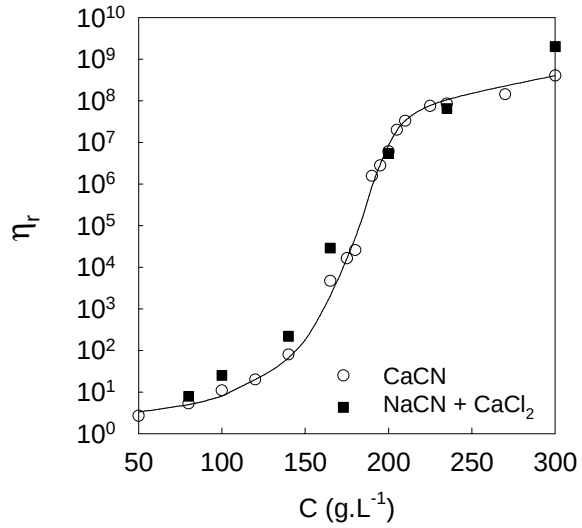

Figure 10. Relative zero shear viscosity (η_r) of mixtures of sodium caseinates and $CaCl_2$ at R=8 compared with that of calcium caseinates as a function of the protein concentration at 20°C.

4. Conclusions

Dense caseinate suspensions are visco-elastic liquids, both when the counter ion is Ca^{2+} or Na^{+}. For both systems the viscosity increases strongly with increasing concentration and decreasing temperature, but the dependence is stronger for CaCN. For NaCN, the increase is mainly caused by jamming of small caseinate particles. For CaCN, attractive interactions are mediated by the Ca^{2+} and induce the formation of dense domains of protein. CaCN suspensions micro-phase separate leading to a heterogeneous structure. The behaviour of CaCN is the same as that of NaCN to which the same amount of Ca^{2+} was added in the form of $CaCl_2$. The behaviour of mixtures of CaCN and NaCN is indistinguishable from that of pure NaCN if less than 50% CaCN is present. If more CaCN is present in the mixture the viscosities are intermediate at high and low protein concentrations, but may be slightly higher than either of the pure systems in a narrow concentration range between 150 and 190 g.L^{-1}.

References.

Bouchoux, A., Debbou, B., Gésan-Guiziou, G., Famelart, M.-H., Doublier, J. L., & Cabane, B. (2009). Rheology and phase behavior of dense casein micelle dispersions. *The Journal of Chemical Physics*, 131, 165-106.

Carr, A. J., Southward, C. R., & Creamer, L. K. (2003). Protein hydration and viscosity of dairy fluids. In P. F. Fox, & P. L. H. McSweeney (Eds.) (3rd ed.). *Proteins: Vol. 1. Advanced dairy chemistry* New York, NY, USA: Kluwer Academic/Plenum Publishers.

Chu, B., Zhou, Z., Wu, G., & Farrell, H. M. J. (1995). Laser light scattering of model casein solutions: effects of high temperature. *Journal of Colloid and Interface Science*, 170, 102-112.

Cuomo, F., Ceglie, A., & Lopez, F. (2011). Temperature dependence of calcium and magnesium induced caseinate precipitation in H_2O and D_2O. *Food Chemistry*,126, 8-14.

Dalgleish, D. G.,&Parker, T. G. (1980). Binding of calcium ions to bovine α_{s1}-casein and precipitability of the protein-calcium ion complexes. *Journal of Dairy Research*, 47,113-122.

Dalgleish, D. G., Paterson, E., & Horne, D. S. (1981). Kinetics of aggregation of α_{s1}-casein/Ca^{2+} mixtures: charge and temperature effects. *Biophysical Chemistry*, 13, 307-314.

Farrell, H. M., Jr., Kumosinski, T. F., Pulaski, P., & Thompson, M. P. (1988). Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization. *Archives of Biochemistry and Biophysics*, 265, 146-158.

Farrer, D., & Lips, A. (1999). On the self-assembly of sodium caseinate. *International Dairy Journal*, 9, 281-286.

Fichtali, J., Van de Voort, F. R., & Doyon, G. J. (1993). A rheological model for sodium caseinate. *Journal of Food Engineering*, 19, 203-211.

Grabowska, K. J., van der Goot, A. J., & Boom, R. M. (2012). Salt-modulated structure formation in a dense calcium caseinate system. *Food Hydrocolloids*, 29, 42-47.

Guo, M., Campbell, B. E., Chen, K., Lenhoff, A. M., & Velev, O. D. (2003). Casein precipitation equilibria in the presence of calciums ions and phosphates. *Colloids and Surfaces B: Biointerfaces*, 29, 297-307.

HadjSadok, A., Pitkowski, A., Benyahia, L., Nicolai, T., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids*, 22, 1460-1466.

Horne, D. S. (1983). Calcium induced precipitation of α_{s1}-casein effect of modification of lysine residues. *International Journal of Biological Macromolecules*, 5, 296-300.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion. Colloid and Interface Science*, 11, 148-153.

Lopez, F., Cuomo, F., Lo Nostro, P., & Ceglie, A. (2012). Effects of solvent and alkaline earth metals on the heat-induced precipitation process of sodium caseinate. *Food Chemistry*, 136, 266-272.

Loveday, S. M., Rao, M. A., Creamer, L. K., & Singh, H. (2010). Rheological behavior of high concentration sodium caseinate dispersions. *Journal of Food Science*, 75, N30-N35.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Food Chemistry*, 48, 1610-1616.

Manski, J. M., van der Goot, A. J., & Boom, R. M. (2007). Formation of fibrous materials from dense calcium caseinate dispersions. *Biomacromolecules*, 8, 1271-1279.

Monedero, F. M., Fabra, M. J., Talens, P., & Chiralt, A. (2010). Effect of calcium and sodium caseinates on physical characteristics of soy protein isolateelipid films. *Journal of Food Engineering*, 97, 228-234.

Mulvihill, D. M., & Fox, P. F. (1989). Physico-chemical and functional properties of milk proteins. InP. F. Fox (Ed.). *Developments in dairy chemistry*, Vol. 4, (pp. 131-172). London, UK: Elsevier Applied Science.

Parker, T. G., & Dalgleish, D. G. (1981). Binding of calcium ions to bovine beta-casein. *Journal of Dairy Research*, 48, 71-76.

Pitkowski, A., Durand, D., & Nicolai, T. (2008). Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science*, 326, 96-102.

Pitkowski, A., Nicolai, T., & Durand, D. (2009). Stability of caseinate solutions in the presence of calcium. *Food Hydrocolloids*, 23, 1164-1168.

Sosa-Herrera, M., Lozano, I., Ponce de León, Y., & Martínez-Padilla, L. (2012). Effect of added calcium chloride on the physicochemical and rheological properties of aqueous mixtures of sodium caseinate/sodium alginate and respective oil-in-water emulsions. *Food Hydrocolloids*, 29, 175-184.

Southward, C. R. (1989). Used of casein and caseinates. InF. P. Fox (Ed.). *Developments in dairy chemistry*, Vol. 4, (pp. 173-244). UK, London: Elsiever Applied Science.

Srinivasan, M., Singh, H., & Munro, P. A. (1999). Adsorption behaviour of sodium and calcium caseinates in oil-in-water emulsions. *International Dairy Journal*, 9, 337-341.

Swaisgood, H. E. (1993). Review and update of casein chemistry. *Journal of Dairy Science*, 76, 3054-3061.

Thomar, P., Durand, D., Benyahia, L., & Nicolai, T. (2012). Slow dynamics and structure in jammed milk protein suspensions. *Faraday Discussions*, 158, 325-339.

Ye, A., Srinivasan, M., & Singh, H. (2000). Influence of NaCl addition on the properties of emulsions formed with commercial calcium caseinate. *Food Chemistry*, 69, 237-244.

Zittle, C. A., & Dellamonica, E. S. (1958). Binding of calcium to casein: influence of pH and calcium and phosphate concentrations. *Archives of Biochemistry and Biophysics*, 76, 342-353.

Chapter 5. The influence of adding monovalent salt on the rheology of concentrated sodium caseinate suspensions and the solubility of calcium caseinate.

The influence of adding monovalent salt on the rheology of concentrated sodium caseinate suspensions and the solubility of calcium caseinate.

Published in *International Dairy Journal (2014)*, 37(1), 48-54.

Peggy Thomar, Lazhar Benyahia, Dominique Durand, Taco Nicolai

LUNAM Université du Maine, IMMM UMR-CNRS 6283, Polymères, Colloïdes et Interfaces, 72085 le Mans Cedex 9, France.

Email : Taco.Nicolai@univ-lemans.fr

Abstract.

The effect of adding NaCl or KCl on the rheology of concentrated sodium caseinate suspensions was investigated over a wide range of protein concentrations (C= 40–235g.L^{-1}) and temperatures (10-60°C). The viscosity increased strongly with increasing protein concentration, due to close-packing of caseinate particles, but decreased with increasing temperature. For C≥80g.L^{-1}, increasing the salt concentration above 0.2 M caused an increase of the viscosity by up to more than two orders of magnitude. Oscillatory shear measurements showed that the suspensions were visco-elastic and that increasing the salt concentration caused an increase of the terminal relaxation time. Calcium ions caused attractive interaction between the caseins and led to the formation of dense protein domains that sedimentated by centrifugation. Addition of NaCl led in this case to dissociation of the dense protein domains and increased the solubility of the caseins.

1. Introduction.

Caseins are the most abundant proteins in milk. They are composed of four types of proteins α_{s1}, α_{s2}, β and κ with a molar mass close to 20kDa (Swaisgood, 1993). In milk, caseins are assembled into aggregates with a radius of about 100 nm called casein micelles that are held together by colloidal calcium phosphate (CCP) (De Kruif, 1998; De Kruif & Holt, 2003; Horne, 2006). CCP can be removed by lowering the pH to the isoelectric point causing precipitation of the caseins. They can be resolubilized in the form of sodium caseinate (NaCas) or calcium caseinate (CaCas) by a neutralization with NaOH or $Ca(OH)_2$, respectively (Mulvihill & Fox, 1989; Southward, 1989). Both sodium and calcium caseinate are widely used as emulsifying, foaming or texturing agents in food products.

The rheology of caseinate suspensions has already been investigated in some detail (Bouchoux, et al., 2009; Farrer & Lips, 1999; Fichtali, Van de Voort, & Doyon, 1993; Loveday, Rao, Creamer, & Singh, 2010). In aqueous solution, NaCas forms small aggregates with a radius of about 10 nm consisting of about 15 molecules (Chu, Zhou, Wu, & Farrell, 1995; HadjSadok, Pitkowski, Benyahia, Nicolai, & Moulai-Mostefa, 2008; Lucey, Srinivasan, Singh, & Munro, 2000). The size of these aggregates depends only weakly on the temperature or the pH, as long as it is higher than 5.2. The viscosity of NaCas solutions increases strongly with increasing protein concentration above about $100g.L^{-1}$ because the particles become close packed (Pitkowski, Durand, & Nicolai, 2008).

In dilute solutions, the molar mass and the hydrodynamic radius of NaCas aggregates were found to increase upon addition of NaCl up to 0.1M, but remained constant at higher NaCl concentrations, at least up to 0.8M (HadjSadok, et al., 2008). In more concentrated systems, the viscosity of NaCas suspensions showed a tendency to increase when NaCl was added (Carr, Southward, & Creamer, 2003; Carr & Munro, 2004; Hermansson, 1975; Konstance & Strange, 1991). Carr and Munro (2004) reported an increase of the shear moduli for NaCas suspensions containing more than 1M NaCl, which they attributed to the reduction of protein hydration.

The presence of calcium ions can strongly influence the properties of caseinate suspensions. κ-casein is insensitive to Ca^{2+} but the other caseins can specifically bind Ca^{2+} reducing the net charge of the proteins and thus the repulsive electrostatic interaction between the particles (Dalgleish & Parker, 1980; Dalgleish, Paterson, & Horne, 1981; Farrell, Kumosinski, Pulaski, & Thompson, 1988). Above a critical concentration of $CaCl_2$, NaCas suspensions become turbid (Thomar, Durand, Benyahia, & Nicolai, 2012) due to the formation of micron-sized

dense proteins domains (Alvarez, Risso, Canales, Pires, & Gatti, 2008; Thomar, et al., 2012). The presence of Ca^{2+} can also strongly modify the rheological properties of caseinate suspensions. Adding a small quantity of $CaCl_2$ causes an increase of the viscosity, whereas a large quantity can cause insolubility and precipitation (Cuomo, Ceglie, & Lopez, 2011; Farrell, et al., 1988; Guo, Campbell, Chen, Lenhoff, & Velev, 2003; Lopez, Cuomo, Lo Nostro, & Ceglie, 2012; Pitkowski, Nicolai, & Durand, 2009; Zittle, DellaMonica, Rudd, & Custer, 1958) of a fraction of the proteins causing a decrease of the viscosity (Carr, Munro, & Campanella, 2002; Thomar, et al., 2012). However, addition of a large quantity of $CaCl_2$ to concentrated caseinate suspensions (>140g.L^{-1}) leads to an increase of the viscosity due to a close-packing of the dense protein domains (Thomar, et al., 2012). The influence of Ca^{2+} on the properties of caseinate suspensions is the same whether they are added in the form of $CaCl_2$ or are present as counterions in the case of CaCas (Thomar, Nicolai, Benyahia, & Durand, 2013).

So far little attention has been paid to the effect of adding monovalent salt to caseinate suspensions that contain Ca^{2+}. It was noted that adding NaCl decreased the turbidity of CaCas suspensions and modified their emulsifying properties (Ye, Srinivasan, & Singh, 2000). Moreover, in the presence of NaCl, less caseinate precipitated after addition of $CaCl_2$ (Ye, et al., 2000; Zittle, DellaMonica, & Custer, 1957), suggesting a competition between Ca^{2+} and Na^{+} for the binding sites of caseinate. Here we report on an investigation of the effect of monovalent salt on the structure and the rheology of NaCas and CaCas suspensions over a wide range of protein concentrations. In the first part we discuss the dynamic mechanical properties of dense NaCas suspensions as a function of the NaCl or the KCl concentration. In the second part we discuss the effect of adding NaCl on the solubility and the heterogeneous structure of caseinate suspensions in the presence of Ca^{2+}.

2. Materials and methods

2.1 Preparation of caseinate solutions. The NaCas and CaCas powders used for this study were acquired from Armor Protein (Saint-Brice, France). The CaCas powder contained 1.3 % (w/w) calcium and 0.3 % (w/w) sodium, whereas the NaCas powder contained 1.3 % (w/w) sodium and no detectable calcium. The powders contained approximately 5% (w/w) water.

The powders were dissolved by adding deionised water (Millipore) while stirring and kept overnight at room temperature while stirring. 3 mM sodium azide was added to the water as a

bacteriostatic agent. Concentrated casein solutions ($C>150g.L^{-1}$) were very viscous at room temperature and needed to be heated at 80°C during 30 minutes while stirring in order to render them fully homogeneous. In this way homogeneous solutions could be prepared up to 300g L^{-1}. Adjustment of the pH to 6.7 was done by slowly adding 0.1M HCl or 1M NaOH while stirring. The casein concentration was determined by measuring the UV absorption at a wavelength of 280nm using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France) with extinction coefficient $0.85L.g^{-1}.cm^{-1}$.

2.2. Rheology. Continuous and oscillatory shear measurements were made with a stress-imposed rheometer (TA Instruments Rheolyst AR2000, New Castle, DE, USA) using a cone - plate geometry (20 mm, 4.01 degrees). Suspensions with $\eta \leq 0.1$Pa.s were measured with another stress-imposed rheometer (MCR 301, Anton-Paar, Graz, Austria) using a Couette geometry (inner and out diameters: 26.6 and 28.9 mm respectively). The temperature was controlled by a Peltier system and the geometry was covered with a mineral oil to prevent water evaporation. The zero-shear viscosity was determined by flow measurements at low shear rates and for $\eta \geq 1$Pa.s by oscillatory measurements at low frequencies. It was verified that the same values were obtained using continuous or oscillatory shear.

2.3. Confocal Laser Scanning Microscopy. Confocal Laser Scanning Microscopy (CLSM) observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany) using a water immersion objective lens HCx PL APO 63x NA=1.2. Fluorochrome rhodamine b was used to label the caseins. A small amount of a concentrated rhodamine solution was added to the casein solutions to yield a total concentration of 5ppm. The rhodamine was excited using a helium-neon laser with wavelength 543nm and the fluorescence was detected with a photomultiplier. Care was taken not to saturate the fluorescence signal and it was verified that the amplitude of the signal was proportional to the protein concentration.

2.4. Calcium activity. The calcium ion activity was determined using a calcium-specific electrode (Fisher Scientific, Hampton, NH, USA). A calibration curve was obtained by measuring the potential of $CaCl_2$ solutions (0-50mM) in water or NaCl. The concentration of free calcium ions was determined by assuming that the activity of bound Ca^{2+} was negligible.

3. Results and Discussions

3.2. Influence of NaCl on the rheology NaCas suspensions

The influence of NaCl on the rheology of NaCas suspensions was studied by measuring the zero shear viscosity and the frequency dependent shear moduli over a wide range of protein concentrations (C= 40-300g.L^{-1}), NaCl concentrations ([NaCl]= 0-4M) and temperatures (T= 10-60°C). Figure 1 shows the zero-shear viscosity of NaCas suspensions at C= 80g.L^{-1} normalized by that of water (η_{rel}), as a function of the NaCl concentration. We note that the concentration of sodium counterions of NaCas is 48mM at C= 80g.L^{-1}. Above a critical concentration of NaCl, which increased with increasing temperature, a sharp increase of η_{rel} was observed before precipitation of the proteins (salting out), which started at 3.5M. The effect of NaCl became significant only above 0.1M NaCl and leveled off at 2.5M NaCl. For a given NaCl concentration, η_{rel} decreased with increasing temperature. This has already been reported for suspensions without added salt or at low salt concentrations and was attributed to a reduction of the effective volume fraction of the caseinate particles with increasing temperature (Pitkowski, et al., 2008; Thomar, et al., 2012).

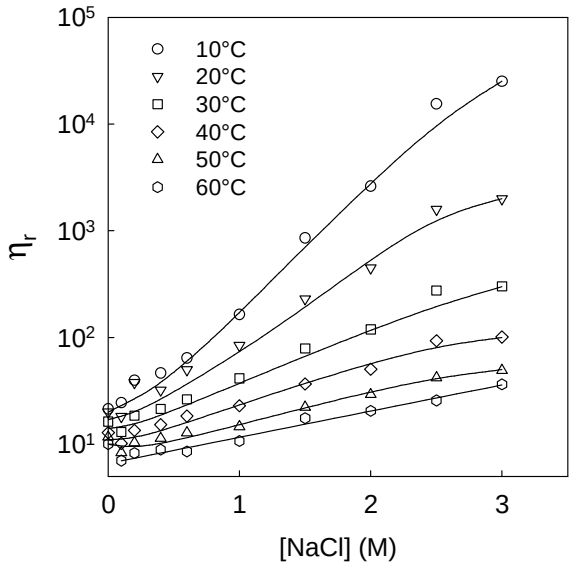

Figure 1. Log-lin representation of the relative zero shear viscosity (η_{rel}) as a function of the NaCl concentration for sodium caseinate suspensions (C=80 g.L^{-1}, pH 6.7) at different temperatures as indicated in the figure.

The effect of the shear rate on the viscosity at different NaCl concentrations is shown in Figure 2. Elsewhere it was shown that dense suspensions of NaCas without added salt were shear thinning (Pitkowski, et al., 2008). Here we find for the more viscous solutions that increasing the shear rate causes initially shear thickening, followed by shear thinning at higher shear rates. The same behavior was observed during repeated shear rate ramps.

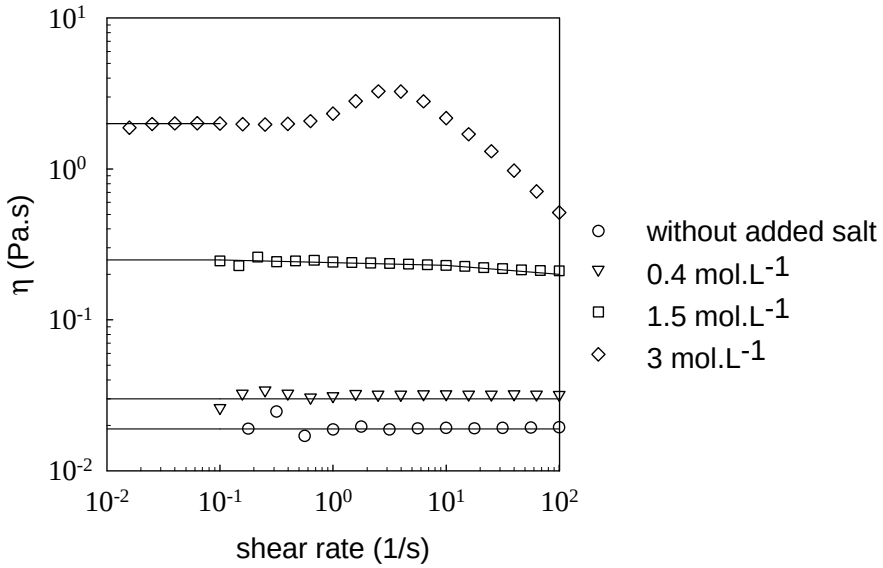

Figure 2. Shear rate dependence of the viscosity (η) of sodium caseinate suspensions (C=80g.L^{-1}, pH 6.7) at 20 °C and for different added NaCl concentrations, as indicated in the figure. The lines are guides to the eye.

Similar effects of NaCl on the viscosity were observed at higher casein concentrations up to 235g.L^{-1}. In Figure 3, we show the concentration dependence of η_{rel} at 20 °C for several high NaCl concentrations. It was already shown that at low salt concentrations that η_{rel} increases strongly with increasing protein concentration when the caseinate particles become close packed (Pitkowski, et al., 2008). Clearly, the increase of the viscosity is much stronger at higher NaCl concentrations and starts at lower protein concentrations. However, for C<70g.L^{-1} the effect of adding NaCl on the viscosity is small even at high salt concentrations. For C>100g.L^{-1}, the relative increase of the viscosity due to addition of NaCl did not depend on the protein concentration and is by more than two orders of magnitude when 3M NaCl is added.

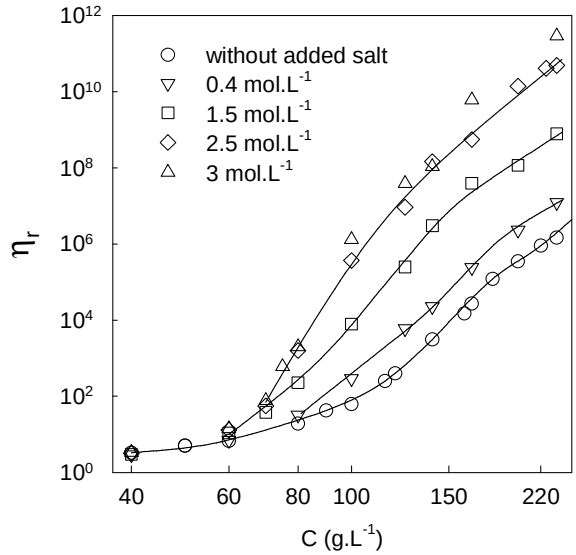

Figure 3. Protein concentration dependence of the relative zero-shear viscosity (η_{rel}) at 20 °C for suspensions of sodium caseinate (pH 6.7) at different NaCl concentrations as indicated in the figure. The lines are guides to the eye.

The frequency dependence of the storage (G') and loss (G") shear moduli of the caseinate suspensions were determined at different temperatures. As was already shown elsewhere for suspensions at low ionic strength, master curves can be obtained by temperature-frequency superposition (Pitkowski, et al., 2008). Figure 4 shows master curves obtained for suspensions at C = 165 g.L^{-1} at different NaCl concentrations with T_{ref} = 10°C. At each ionic strength the frequency dependence of G' and G" is typical for visco-elastic systems with a solid-like response at high frequencies and a liquid-like response at low frequencies. The transition between the two behaviours shifts to lower frequencies when more salt is present.

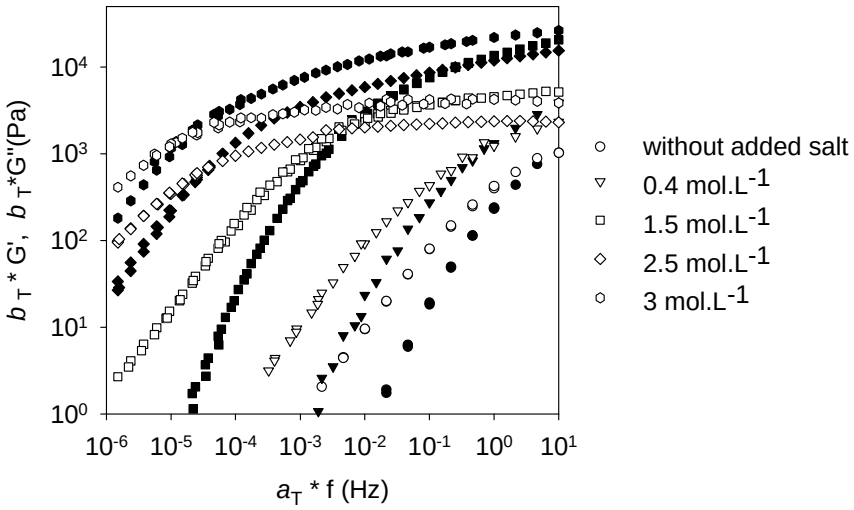

Figure 4.Master curves (T_{ref}=10 °C) obtained from a temperature-frequency superposition of the frequency dependent elastic G' (filled symbols) and the viscous G" (opened symbols) shear moduli for suspensions of sodium caseinate (C=165 g L^{-1}, pH 6.7) for different concentrations of NaCl as indicated in the figure.

The terminal relaxation time (τ) was calculated from the frequency (f_c) where G' and G" crossed: $\tau = 1/(2\pi f_c)$ and the high frequency elastic shear modulus (G_e) was taken rather arbitrarily as 10 times the value of G' at f_c. The concentration dependence of τ and G_e at different NaCl concentrations is shown in Figure 5.

At the two higher NaCl concentrations, G_e increased sharply with increasing protein concentration between C = 80 and 150 g.L^{-1} and weakly at higher concentrations, see Figure 5a. At lower salt concentrations, G_e could only be determined with some precision for C≥140g.L^{-1}. At a given protein concentration, G_e did not depend strongly on the NaCl concentration.

Figure 5b shows that at all NaCl concentrations, τ increased strongly with increasing protein concentration over the whole range where it could be determined. Contrary to the elastic modulus, the relaxation time depended strongly on the NaCl concentration. The increase of the viscosity with increasing NaCl concentration is thus principally caused by an increase of the relaxation time.

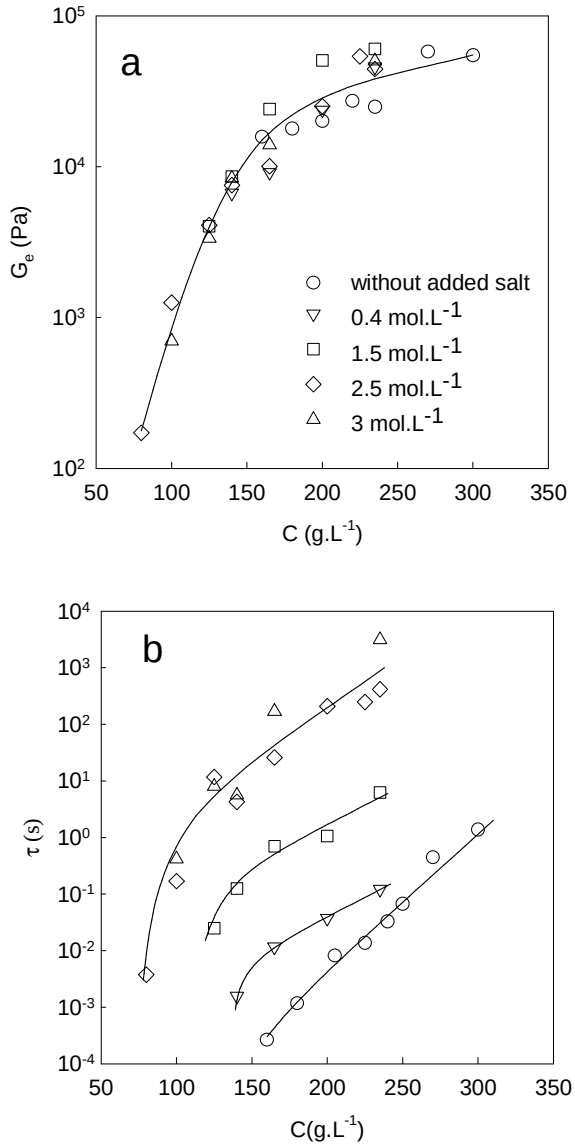

Figure 5a.Elastic modulus (G_e) of sodium caseinate suspensions (pH 6.7) as a function of the protein concentration at 10 °C for different concentrations of NaCl as indicated in the figure. The solid line is a guide to the eye.

Figure 5b.Protein concentration dependence of the terminal relaxation time of sodium caseinate suspensions at 10 °C for different concentrations of NaCl as indicated in Figure 5a. The solid lines are guides to the eye.

For dense suspensions of colloidal particles the terminal relaxation time typically corresponds to the time needed for the particles to escape from the cage formed by their neighbours. The escape time increases when the effective volume fraction of the particles increases, but also when the attraction between the particles increases (Dawson 2002; Hunter and Weeks 2012). The former effect explains the strong increase of τ with increasing protein concentration. It may also explain in part why the relaxation time, and thus the viscosity, drops with increasing

temperature (Thomar, et al., 2012). However, the strong increase of τ with increasing NaCl concentration is most likely caused by increasing attractive interactions between the particles. Electrostatic interaction is already efficiently screened at 0.2M NaCl where the viscosity is still close to that in pure water (Carr, et al., 2002). Therefore we may exclude screening as the origin of the increase of the viscosity at high NaCl concentrations. Most likely the increase of the attractive interaction between the proteins is caused by partial dehydration of the proteins.

We repeated some of the measurements with KCl. The protein concentration dependence of η_{rel} at 20 °C is shown in Figure 6 at 0.4 and 1.5M KCl and compared with the results for NaCl. Clearly, the effect of adding KCl is the same as that of adding of NaCl within the experimental error. We also observed salting out with KCl at 4M. These results show that the phenomena discussed here are not specific for sodium.

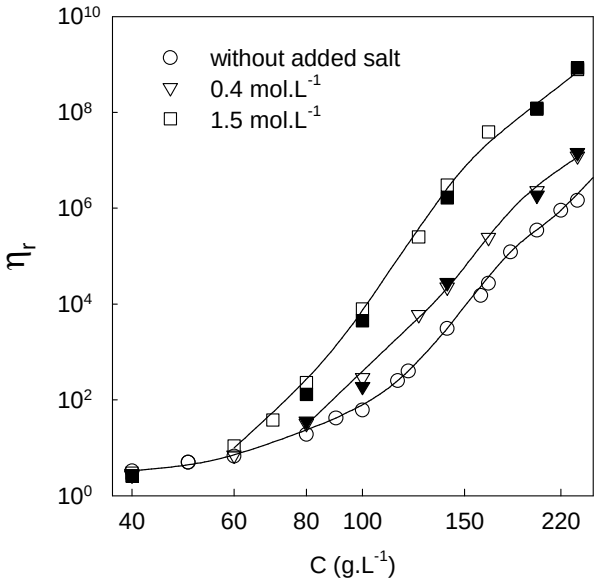

Figure 6.Concentration dependence of the relative zero shear viscosity (η_{rel}) at 20°C of suspensions of sodium caseinate (pH 6.7) for different NaCl (open symbols) and KCl (closed symbols) concentrations as indicated in the figure.

3.2. Influence of NaCl on the calcium induced precipitation of casein.

As mentioned in the introduction, adding calcium to NaCas suspensions leads to the formation of dense protein particles with a radius of about a micron, which affects the structure and the rheology of the suspensions. The effect is determined by the concentration of calcium ions ($[Ca^{2+}]$) and it is equivalent to use CaCas or NaCas with added $CaCl_2$ (Thomar, et al., 2013). For not too viscous suspensions ($C < 100 g.L^{-1}$), the large dense protein particles can be removed

by centrifugation (50,000 *g* – 2 h) and a clear supernatant is obtained. It was already shown elsewhere (Pitkowski, et al., 2009; Thomar, et al., 2012) that in the absence of NaCl the fraction of soluble proteins (F) in the supernatant decreases with increasing [Ca^{2+}].

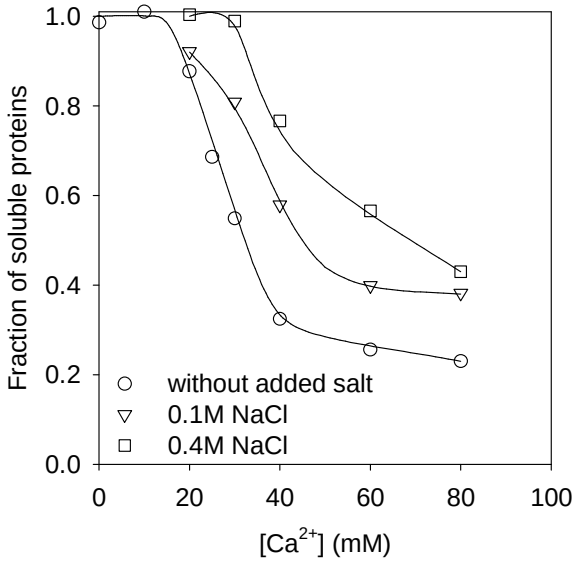

Figure 7.Fraction of soluble proteins in a suspension of sodium caseinate (C=80g L^{-1}, pH6.7) as a function of the total calcium ion concentration for different concentrations of NaCl at 20°C, as indicated in the figure.

Figure 7 compares the effect of Ca^{2+} on the solubility of a NaCas suspension at C = 80g.L^{-1}in water with that obtained in the presence of 0.1 or 0.4M NaCl. It is clear that adding NaCl increases the solubility of caseinate at a given Ca^{2+} concentration. Notice that at these salt concentrations the effect of NaCl on hydration of the proteins is still relatively weak. The dependence of F on the NaCl concentration is shown in Figure 8 at two $CaCl_2$ concentrations: 28 and 40mM. As was mentioned above, the concentration of sodium counterions at C=80 g.L^{-1} is 48mM and cannot be neglected at low concentrations of added NaCl. For both systems the solubility increased with increasing NaCl concentration up to 2M, above which it decreased sharply due to salting out. Notice that the effect of salting out started at a lower NaCl concentrations in the presence of Ca^{2+} than for pure NaCas. At 28mM $CaCl_2$ no caseinate precipitated between 0.4 and 2M NaCl, whereas at 40mM $CaCl_2$ a small fraction of caseinate precipitated even at 2M NaCl.

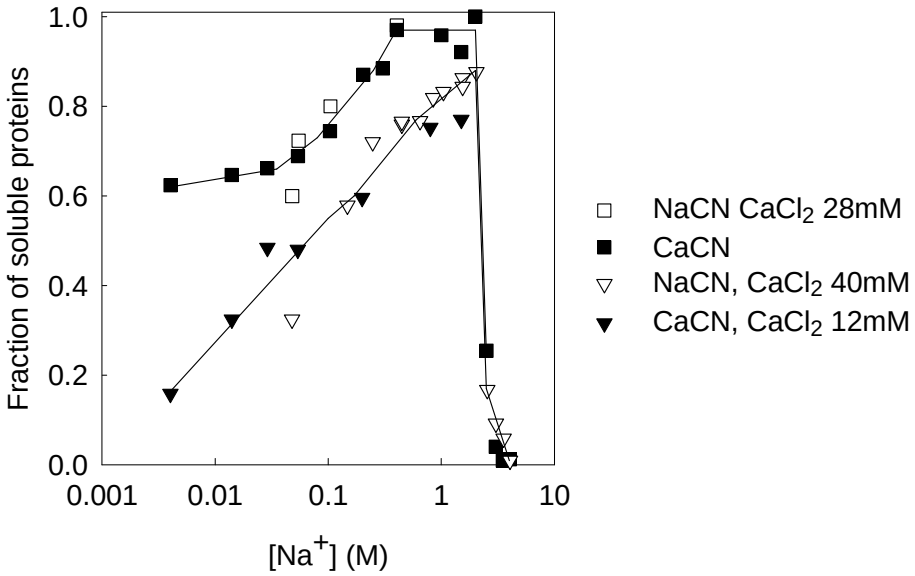

Figure 8. Fraction of soluble proteins in suspensions of sodium and calcium caseinate (80g.L^{-1}, pH6.7) at 20°C as a function of the Na^+ concentration at different concentrations of $CaCl_2$.

In order to verify that the solubility was controlled by the Ca^{2+} concentration also when it is present in the form of counterions, we investigated CaCas suspensions with the same total calcium ion concentration. As mentioned above, the calcium counterion concentration of CaCas is 28mM so that pure CaCas can be compared with NaCas containing 28mM $CaCl_2$, while for the comparison with NaCas containing 40mM $CaCl_2$ we added 12mM $CaCl_2$ to the CaCas suspension. Figure 8 shows that the effect of adding NaCl on the solubility, is indeed the same in agreement with earlier results (Thomar, et al., 2013) on solutions without NaCl (Thomar, et al., 2013).

The origin of the increased solubility of casein upon addition of NaCl is most likely related to the reduction of Ca^{2+} binding by casein when Na^+ competes for the binding sites of Ca^{2+}. Dalgleish and Parker (1980) reported for pure α_{s1}-casein solutions a decrease of bound Ca^{2+} by adding NaCl up to 0.2M. For pure β-casein, a decrease of bound Ca^{2+} was also reported by adding NaCl up to 0.1M (Baumy & Brulé, 1988). We have measured the activity of Ca^{2+} using a calcium sensitive electrode for a CaCas suspension at 80g.L^{-1}, see Figure 9. In pure water, almost all Ca^{2+} was bound to the proteins. With increasing NaCl concentration an increasing fraction of Ca^{2+} was released into solution, but the effect of adding more NaCl stagnated at 1M when approximately half of the bound Ca^{2+} had been released. Apparently about half of the calcium counterions is more strongly bound and is not released even in a large excess of Na^+.

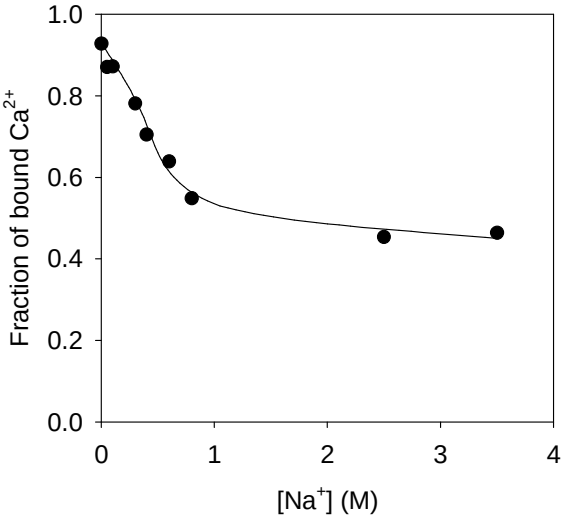

Figure 9. Fraction of bound Ca^{2+} as a function of the NaCl concentration for suspensions of calcium caseinate (C=80 g.L^{-1}, pH 6.7) at 20°C. The total concentration of Ca^{2+} was 28 mM.

We have investigated the structure of the caseinate suspensions with CLSM. In an earlier study we showed that above a critical number of Ca^{2+} ions per casein molecule the suspensions become heterogeneous on microscopic length scales (Thomar, et al., 2012). Here we investigate the influence of adding NaCl on the structure. Images of NaCas suspensions at C= 80g.L^{-1} containing 40mM of $CaCl_2$ and different concentration of NaCl are shown in Figure 10. In the absence of NaCl the suspensions contained dense protein domains. When an increasing amount of NaCl was added fewer but larger domains were formed until at 1.5M NaCl the suspension became homogeneous. Adding even more NaCl led again to the formation of large dense protein domains (Figure 10) which we attribute to salting out. A similar effect of adding NaCl was observed for suspensions of 80g.L^{-1} CaCas that contained 28mM Ca^{2+} in the form of counterions, results not shown. However, this latter system became homogeneous at a lower NaCl concentration (0.4M), because it contained less Ca^{2+}. The reduction of the number of dense domains with increasing NaCl concentration is consisted with the increase of the solubility discussed above and explains the decrease of the turbidity that was reported by Ye et al. (2000).

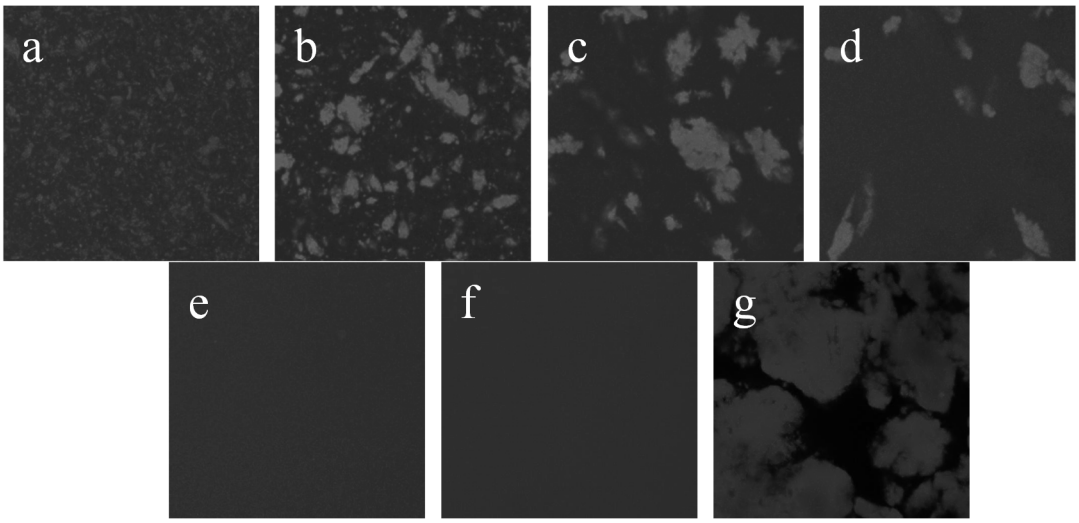

Figure 10. CLSM images (160x160 µm) of suspensions of sodium caseinate (C=80g.L^{-1}, pH 6.7, 20°C) containing 40mM $CaCl_2$ and various concentrations of NaCl: (a) without added salt; (b) 0.2M NaCl; (c) 0.4M NaCl; (d) 1M NaCl; (e) 1.5M NaCl; (f) 2M NaCl; (g) 3M NaCl. The contrast has been artificially increased.

Adding NaCl may thus be considered as equivalent to removing a fraction of casein-bound Ca^{2+}. In pure water, caseinate is fully soluble at C=80g.L^{-1} as long as the concentration of bound Ca^{2+} ions is less than 20mM (Figure 7). This means that in order to fully solubilize suspensions at [Ca^{2+}] = 40mM, half of the bound calcium ions need be liberated, which can almost be realized by adding 2 M NaCl. On the other hand, when the suspension contains 28mM Ca^{2+} only about a third of the calcium ions needs to be liberated which can be achieved by adding 0.4M NaCl.

4. Conclusions

Addition of high concentrations of NaCl or KCl to caseinate solutions leads to dehydration of caseins, which causes attractive interaction between small caseinate particles. Above 0.2M NaCl the viscosity of concentrated caseinate suspensions (C> 70 g.L^{-1}) increases strongly with increasing salt concentration until they precipitate at 4M. The increase was observed over wide range of temperatures and protein concentrations. Concentrated caseinate suspensions are viscoelastic liquids with a high frequency elastic modulus that increases strongly with the protein concentration, but depends only weakly on the salt concentration. The terminal relaxation time of the suspensions increases strongly with increasing salt concentration causing a strong increase of the viscosity. Probably, dehydration leads to transient bond formation

between caseinate particles which increases the time needed for the particles to escape from the cage formed by their neighbouring particles in concentrated suspensions. Calcium ions bind specifically to caseinate, which causes the formation of dense protein domains above a critical ratio of calcium to protein that precipitate when centrifuged. High concentrations of Na^+ can displace up to 50% of the calcium ions bound to the caseins. As a consequence, the calcium induced heterogeneity of caseinate suspensions and their reduced solubility are mitigated by addition of NaCl.

References

Alvarez, E. M., Risso, P. H., Canales, M. A. M., Pires, M. S., & Gatti, C. A. (2008). Hydrodynamic properties-structure relationship for sodium caseinates in presence of calcium. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 327, 51-56.

Baumy, J. J., & Brulé, G. (1988). Effect of pH and ionic strength on the binding of bivalent cations to beta casein. *Le Lait*, 68, 409-418.

Bouchoux, A., Debbou, B., Gesan-Guiziou, G., Famelart, M.-H., Doublier, J.-L., & Cabane, B. (2009). Rheology and phase behavior of dense casein micelle dispersions. *The Journal of Chemical Physics*, 131, 165106.

Carr, A. J., Munro, P. A., & Campanella, O. H. (2002). Effect of added monovalent or divalent cations on the rheology of sodium caseinate solutions. *Journal of Dairy Research*, 12, 487-492.

Carr, A. J., Southward, C. R., & Creamer, L. K. (2003). Protein hydration and viscosity of dairy fluids. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry, Vol. 1, Proteins. 3rd ed* (pp. 1289-1323). New York, NY, USA: Kluwer Academic/Plenum Publishers.

Carr, A. J., & Munro, P. A. (2004). Reversible cold gelation of sodium caseinate solutions with added salt. *Journal of Dairy Research*, 71, 126-128.

Chu, B., Zhou, Z., Wu, G., & Farrell, H. M. J. (1995). Laser light scattering of model casein solutions: effects of high temperature. *Journal of Colloid and Interface Science*, 170, 102-112.

Cuomo, F., Ceglie, A., & Lopez, F. (2011). Temperature dependence of calcium and magnesium induced caseinate precipitation in H_2O and D_2O. *Food Chemistry*, 126, 8-14.

Dalgleish, D. G., & Parker, T. G. (1980). Binding of calcium ions to bovine α_{s1}-casein and precipitability of the protein-calcium ion complexes. *Journal of Dairy Research*, 47, 113-122.

Dalgleish, D. G., Paterson, E., & Horne, D. S. (1981). Kinetics of aggregation of α_{s1}-casein / Ca^{2+} mixtures: charge and temperature effects. *Biophysical Chemistry*, 13, 307-314.

Dawson, K. A. (2002). The glass paradigm for colloidal glasses, gels, and other arrested states driven by attractive interactions. *Current Opinion in Colloid and Interface Science*, 7, 218-227.

De Kruif, C. (1998). Supra-aggregates of casein micelles as a prelude to coagulation. *Journal of Dairy Science*, 81, 3019-3028.

De Kruif, C., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry, Vol. 1, Proteins*. 3rd ed (pp. 233-276). New York, NY, USA: Kluwer Academic/Plenum Publishers.

Farrell, H. M., Kumosinski, T. F., Pulaski, P., & Thompson, M. P. (1988). Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization. *Archives of Biochemistry and Biophysics*, 265, 146-158.

Farrer, D., & Lips, A. (1999). On the self-assembly of sodium caseinate. *International Dairy Journal*, 9, 281-286.

Fichtali, J., Van de Voort, F., & Doyon, G. (1993). A rheological model for sodium caseinate. *Journal of Food Engineering*, 19, 203-211.

Guo, M., Campbell, B. E., Chen, K., Lenhoff, A. M., & Velev, O. D. (2003). Casein precipitation equilibria in the presence of calciums ions and phosphates. *Colloids and Surfaces B: Biointerfaces*, 29, 297-307.

HadjSadok, A., Pitkowski, A., Benyahia, L., Nicolai, T., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids*, 22, 1460-1466.

Hermansson, A. M. (1975). Functional properties of proteins for food-flow properties. *Journal of Texture Studies*, 5, 425-439.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion in Colloid & Interface Science*, 11, 148-153.

Hunter, G. L., & Weeks, E. R. (2012). The physics of the colloidal glass transition. *Reports on Progress in Physics*, 75, 066501.

Konstance, R., & Strange, E. (1991). Solubility and viscous properties of casein and caseinates. *Journal of Food Science*, 56, 556-559.

Lopez, F., Cuomo, F., Lo Nostro, P., & Ceglie, A. (2012). Effects of solvent and alkaline earth metals on the heat-induced precipitation process of sodium caseinate. *Food Chemistry*, 136, 266-272.

Loveday, S. M., Rao, M. A., Creamer, L. K., & Singh, H. (2010). Rheological behavior of high concentration sodium caseinate dispersions. *Journal of Food Science*, 75, N30-N35.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Food Chemistry*, 48, 1610-1616.

Mulvihill, D. M., & Fox, P. F. (1989). Physico-chemical and functional properties of milk proteins. In P. F. Fox (Ed.), *Developments in Dairy Chemistry* (Vol. 4, pp. 131-172). London, UK: Elsevier Applied Science.

Pitkowski, A., Durand, D., & Nicolai, T. (2008). Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science*, 326, 96-102.

Pitkowski, A., Nicolai, T., & Durand, D. (2009). Stability of caseinate solutions in the presence of calcium. *Food Hydrocolloids*, 4, 1164-1168.

Southward, C. R. (1989). Used of casein and caseinates. In F. P. Fox (Ed.), *Developments in Dairy Chemistry* (Vol. 4, pp. 173-244). London, UK: Elsiever Applied Science.

Swaisgood, H. E. (1993). Review and update of casein chemistry. *Journal of Dairy Science*, 76, 3054-3061.

Thomar, P., Durand, D., Benyahia, L., & Nicolai, T. (2012). Slow dynamics and structure in jammed milk protein suspensions. *Faraday Discussions*, 158, 325-339.

Thomar, P., Nicolai, T., Benyahia, L., & Durand, D. (2013). Comparative study of the rheology and the structure of sodium and calcium caseinate solutions. *International Dairy Journal*, 31, 100-106.

Ye, A., Srinivasan, M., & Singh, H. (2000). Influence of NaCl addition on the properties of emulsions formed with commercial calcium caseinate. *Food Chemistry*, 69, 237-244.

Zittle, C., DellaMonica, E., & Custer, J. (1957). Effect of certain salts on precipitation of casein by calcium chloride and heat. *Journal of Dairy Science*, 40, 280-288.

Zittle, C., DellaMonica, E., Rudd, R., & Custer, J. (1958). Binding of calcium to casein: influence of pH and calcium and phosphate concentrations. *Archives of Biochemistry and Biophysics*, 76, 342-353.

Chapter 6. Dissociation of native casein micelles induced by sodium caseinate.

Dissociation of native casein micelles induced by sodium caseinate.

Published in *Food Hydrocolloids (2015)*, 49, 224-231

Peggy Thomar, Taco Nicolai.

Université du Maine, IMMM UMR-CNRS 6283, Polymères, Colloïdes et Interfaces, 72085 le Mans Cedex 9, France.

Email : Taco.Nicolai@univ-lemans.fr

Abstract.

The effect of addition of sodium caseinate (NaCas) to aqueous solutions of casein micelles was studied at pH 6.7. Addition of NaCas leads to reduction of the turbidity and to a decrease of the fraction of proteins that precipitates during ultracentrifugation. The calcium and orthophosphate concentrations in the supernatant were found to increase with increasing NaCas concentration. The results show that addition of NaCas causes release of colloidal calcium phosphate and dissociation of native caseine micelles. The process is controlled by the weight fraction of NaCas in the mixture and dissociation is complete when the fraction exceeds 75%. The efficacy of NaCas as chelating agent for casein micelles is compared with that of polyphosphate reported in the literature. The liberated calcium and orthophosphate are bound to the added NaCas, which leads to aggregation at higher casein concentrations.

1.Introduction

Casein is the most abundant protein of milk and consists of mainly four different types: α_{s1}, α_{s2}, β and κ in different proportions (Morris, 2002). In milk, casein is associated into an approximately spherical particle with a radius of roughly 100nm that is called casein micelle (Holt, 1992; Horne, 2006; Schmidt, 1982). Casein micelles contain colloidal calcium phosphate (CCP) in the form of nanoclusters (De Kruif & Holt, 2003; Holt, 1982; McMahon & Brown, 1984) to which the caseins are connected via phosphoserine (P-ser) units (Horne, 2006). CCP maintains the integrity of the casein micelles together with hydrophobic interactions and hydrogen bonds between caseins (Dalgleish, 2011; Farrell, Malin, Brown, & Qi, 2006; Holt, 1992, 1998).

At neutral pH, casein micelles dissociate after removal of the CCP by dialysis (Aoki, Yamada, Kako, & Imamura, 1988; Hansen, et al., 1996; Stothart & Cebula, 1982), or addition of calcium-chelating agents like EDTA (Griffin, Lyster, & Price, 1988; Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Marchin, Putaux, Pignon, & Léonil, 2007; Pitkowski, Nicolai, & Durand, 2007), citrate (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Kaliappan & Lucey, 2011; Mizuno & Lucey, 2005), or polyphosphate (de Kort, et al., 2011; Mizuno, et al., 2005; Pitkowski, et al., 2007). After removal of the CCP the casein molecules assemble into small particles with a radius of about 10nm, that are sometimes called submicelles (Hansen, et al., 1996; Panouillé, Durand, Nicolai, Larquet, & Boisset, 2005; Panouillé, Nicolai, & Durand, 2004; Stothart, et al., 1982). A consequence of the dissociation of casein micelles is that the system scatters less light so that the turbidity decreases (Pitkowski, et al., 2007).

Sodium caseinate (NaCas) is obtained by precipitation of casein micelles at pH 4.6 and washing, which removes the calcium phosphate, followed by addition of NaOH to resolubilize the proteins (Mulvihill & Fox, 1989). At neutral pH, NaCas forms particles with a radius of about 10nm depending on the ionic strength (Alvarez, Risso, Canales, Pires, & Gatti, 2008; Chu, Zhou, Wu, & Farrell, 1995; Farrell, et al., 1996; Farrer & Lips, 1999; HadjSadok, Pitkowski, Benyahia, Nicolai, & Moulai-Mostefa, 2008; Lucey, Srinivasan, Singh, & Munro, 2000). NaCas strongly binds calcium ions (Alvarez, et al., 2008; Dalgleish & Parker, 1980; Dalgleish, Paterson, & Horne, 1981; Farrell Jr, Kumosinski, Pulaski, & Thompson, 1988; Parker & Dalgleish, 1981; Zittle & Dellamonica, 1958), which above a critical concentration of calcium ions leads to aggregation and decreased solubility (Guo, Campbell, Chen, Lenhoff, & Velev,

2003; Pitkowski, Nicolai, & Durand, 2009; Thomar, Durand, Benyahia, & Nicolai, 2012). NaCas may thus be considered as a calcium-chelating agent.

Here we address the question as to what extent addition of NaCas to aqueous solutions of casein micelles leads to release of CCP from the micelles and dissociation of the micelles. A second issue is how the redistributed calcium and phosphate influence the properties of the caseins in solution. These issues have to our knowledge not yet been addressed in the literature. They are not only interesting from a fundamental point of view, but are also important for applications in the dairy industry when NaCas is added to milk for processing (Gaygadzhiev, Massel, Alexander, & Corredig, 2012; Nair & Corredig, 2015).

2. Material and Methods

2.1.Materials. Casein micelles in the form of native phosphocaseinate powder (NPCP) was provided by INRA-STLO, Rennes. NPCP was obtained by microfiltration of skimmed milk as reported in Schuck, et al. (1994). The batch used for this study was composed of 83% (w/w) of protein (TNC, Kjeldahl), and contained 2.6 % (w/w) of calcium and 1.7% (w/w) of organic and inorganic phosphorus. The sodium caseinate (NaCas) powder used for this study (Lactonat EN, Lactoprot, Kaltenkirchen, Germany) contained 90% (w/w) protein (TNC, Kjeldahl), 1.3% (w/w) of sodium and 0.7% (w/w) of phosphorus.

2.2. Casein composition. The casein composition of the samples was obtained using reverse phase high pressure liquid chromatography (RP-HPLC). Figure 1 shows the RP-HPLC chromatograms for the NaCas and the NPCP samples used for this study. The positions of the different caseins have been identified by comparing with purified caseins. The fractions of α_{s1}-, α_{s2}-, β-, and κ-casein in NaCas were 34, 3, 44, 9%, respectively. In addition, the sample contained 9% unidentified protein, most likely partially degraded casein. The fractions of α_{s1}-, α_{s2}-, β-, and κ-casein in NPCP were 24, 4, 36, 9%, respectively. This sample contained a relatively large fraction of unidentified casein (27%).

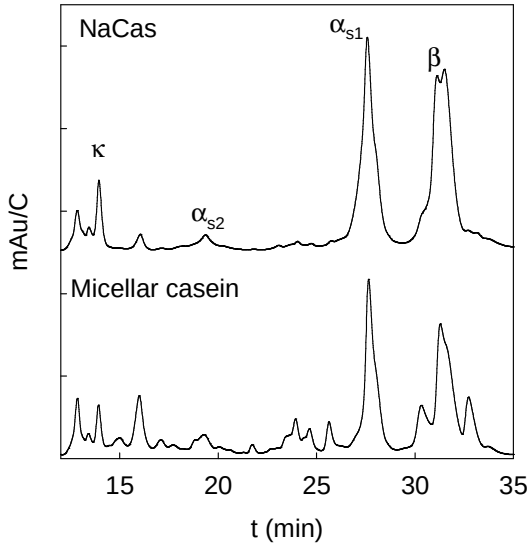

Figure 1. RP-HPLC chromatograms of micellar casein at 15g.L^{-1} and NaCas at 77g.L^{-1}.

2.3. Sample preparation. The powders were hydrated with milliQ water containing 3mM of sodium azide (NaN_3), as bacteriostatic agent. Homogeneous dispersions of NPCP were obtained after keeping the system at 50°C during 16h, while homogeneous dispersions of NaCas were obtained after heating at 80°C during 30 minutes. The pH was adjusted at 20°C to 6.7 by addition of aliquots of HCl or NaOH solutions (1M). The protein concentration was determined by absorption of UV-light with wavelength 280nm (Varian Cary-50 Bio, Les Ulis, France) using an extinction coefficient of 0.81L.g^{-1}.cm^{-1}. NaCas solutions contained a small amount of residual fat globules that were removed by centrifugation (5.10^4*g*; 2h). The loss of protein by this procedure was less than 5%. Mixtures were prepared by mixing the two stock solutions with known protein concentrations determined by UV-adsorbtion and diluting with milliQ water to the required amount.

2.4. Determination of the fraction of sedimentable casein. Solutions were centrifugated at 5.10^4*g* during 2 hours at 20°C using an ultracentrifuge (Beckman Coulter, Allegra 64R, Villepinte, France). We checked different centrifugation times and speeds and found that at the conditions used were largely sufficient to sediment all casein micelles, but none of the NaCas. Subsequently, the protein concentration of the supernatant was determined by UV spectroscopy from which the fraction of sedimentable casein was calculated.

2.5. Determination of the calcium content. The concentration of calcium was determined by flame spectroscopy at 422.7nm (Varian AA240FS, Les Ulis, France). Before measurement the

solutions were diluted in milliQ water to be within the calibration range that was prepared with known concentrations of $CaCl_2$ in milliQ water.

2.6. Determination of the phosphorus content. 1g of sample was dried at 100°C during 5h and at 500°C until dry ashes were obtained. The ashes were suspended in 1M HCl and diluted with water to $1g.L^{-1}$. The dilute sample was dissolved in water containing sodium molybdate and ascorbic acid with final concentrations of $0.125g.L^{-1}$ sodium molybdate and $0.1g.L^{-1}$ ascorbic acid. Total phosphorus was determined by UV spectroscopy at 820nm using a calibration curve that was made with potassium orthophosphate (KH_2PO_4) solutions containing sodium molybdate and ascorbic acid at final concentrations of $0.125g.L^{-1}$ and $0.1g.L^{-1}$.

2.7. RP-HPLC. The different types of caseins were determined by using reverse phase high pressure liquid chromatography (Ultimate 3000, Dionex) with an analytical column C5 Jupiter (250 x 4.6 mm), 30nm pore size, 5µm particle size (Phenomenex, Torrance, USA), see (Miranda, Mahé, Leroux, & Martin, 2004; Visser, Slangen, & Rollema, 1991). Following a procedure described by (Miranda, et al., 2004), the caseins were diluted at a concentration of at 0.4%(w/w) in 0.1 M bis-Tris buffer at pH 8.0, containing 8M urea, 1.3% trisodium citrate and 0.3% DTT. After filtration through 0.45µm pore size filters, 2µL of the solution was injected. The mobile phase was composed of deionised water and trifluoroacetic acid with a gradient of acetonitrile. The UV absorption was detected at 214 nm.

2.8. Turbidity measurements. The turbidity (τ) was measured by spectrophotometry as a function of the wavelength (400-1100nm) as reported by (Pitkowski, et al., 2007). The spectrophotometer (Varian Cary-50 Bio, Les Ulis, France) was coupled with a thermostated bath. The temperature was probed in the samples.

3. Results

3.1. Stability of casein micelles in pure water

Before discussing the effect of adding NaCas to aqueous solutions of casein micelles we need to investigate the stability of the casein micelles in pure water at pH 6.7. A straightforward method to determine the stability is to measure the turbidity, which is directly linked to the molar mass and structure of particles in solution. Solutions of NPCP were prepared at different concentrations by dilution of a stock solution at $C=80g.L^{-1}$. The turbidity was measured

immediately after preparation as a function of the wavelength. Power law dependence of the turbidity (τ) as a function of the wavelength (λ) was observed: $\tau \propto \lambda^{-2.9}$, independent of the casein concentration in agreement with results reported by (Pitkowski, et al., 2007). For a given wavelength the turbidity increased linearly with the concentration up to C= 20 g.L^{-1}: $\tau = 0.6C$ (results not shown), which is close to the dependence reported by Pitkowski et al., (2007) ($\tau = 0.65C$). At higher proteins concentrations the increase was weaker due to interactions between the casein micelles.

The stability of the casein micelles in pure water was studied by measuring the turbidity at τ=685nm as a function of time for different concentrations between 0.4 and 40 g.L^{-1}. The turbidity normalized by the initial value (τ_n) decreased with time, see Figure 2, which shows that casein micelles were not stable in pure water. In milk the CCP within the micelles is in equilibrium with the minerals in the milk serum, but in pure water it dissolves leading to dissociation of the micelles. However, dissolution of CCP increases the mineral content of the water phase which slows down further dissolution of CCP. For this reason the decrease of τ_n was much slower at higher casein concentrations. At the final time of the experiment (16h), micelles at C=0.4g.L^{-1} had completely dissociated whereas at C=40 g.L^{-1} the turbidity had decreased by only 6%.

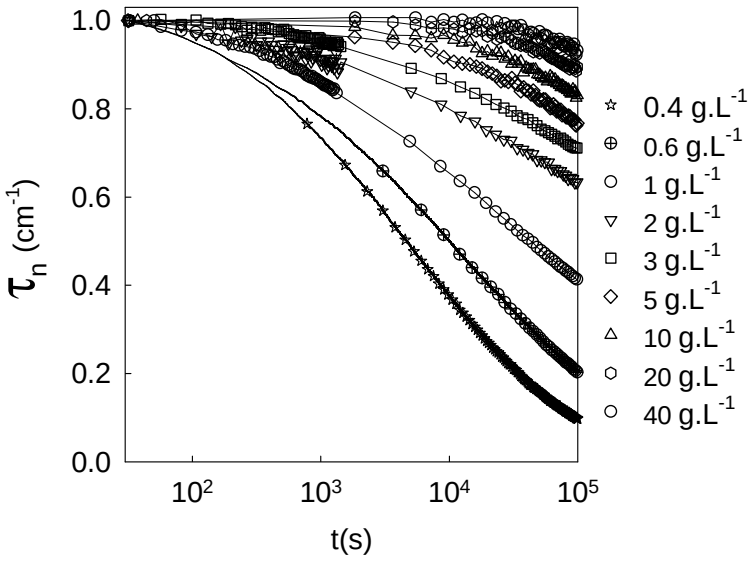

Figure 2. Turbidity at 685nm normalized by the initial value as a function of the time for NPCP suspensions in pure water at different concentrations indicated in the figure.

The solutions were centrifuged 16h after preparation and the protein concentration in the supernatant was determined as described in the materials and methods section. It was found to increase in proportion to the decrease of the turbidity, which confirms that the reduction of the turbidity was caused by dissociation of the micelles as the turbidity of the supernatant was negligible. Remarkably, even at high NPCP concentrations about 15% of the casein remained in the supernatant, whereas the turbidity had only decreased by about 5%. This means that about 10% of the casein was not in the form of micelles immediately in freshly prepared NPCP solutions and a further 5% dissociated during the 16h after preparation.

The casein composition of the supernatant was different from the overall composition. The RP-HPLC chromatogram of the supernatant of a sample at C=15g.L^{-1} 16h after preparation is shown in Figure 3. The protein concentration in the supernatant for this sample was 3 g.L^{-1}. Comparison with the chromatogram of the powder (Figure 1) shows that the fraction of κ-casein and unattributed casein was much higher in the supernatant, while the fraction of α_s-casein in the supernatant was very small. An increase of the κ-casein fraction and a decrease of the α_s-casein fractions might be expected as the latter are rich in phosphoserines, while the former contains only one phosphoserine. The strong increase of the fraction of degraded caseins shows that these caseins were more weakly bound to the casein micelles than α_{s1}- and β-casein.

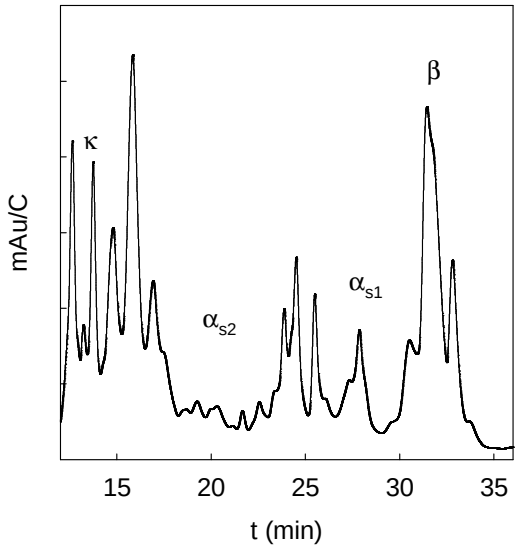

Figure 3. RP-HPLC chromatograph of the supernatant of a suspension of 15 g.L^{-1} NPCP in pure water.

3.2. Characterization of mixtures of casein micelles and sodium caseinate

3.2.1. Dissociation of the casein micelles

In first instance mixtures were studied that contained a fixed amount of 15g.L^{-1} NPCP with different concentrations of NaCas at pH 6.7. The mixtures were centrifuged 16 h after preparation and the concentration of protein in the supernatant was determined as described in the material and methods section. At the centrifugation speed used here, casein micelles sediment, but NaCas does not. Figure 4 shows the weight fraction of non-sedimentable proteins (F) as a function of the weight fraction of NaCas in the mixtures (R). The corresponding ratio of NaCas to casein micelles is equal to 1/(R-1). For pure NPCP (R=0) F was 0.2, because 10% of the micelles was dissociated during preparation and another 10% during ageing for 16h. With increasing NaCas concentration F increased until for R>0.8 almost all casein remained in the supernatant. These results imply that all casein micelles non-sedimentable, because they were dissociated by the presence of NaCas in large excess.

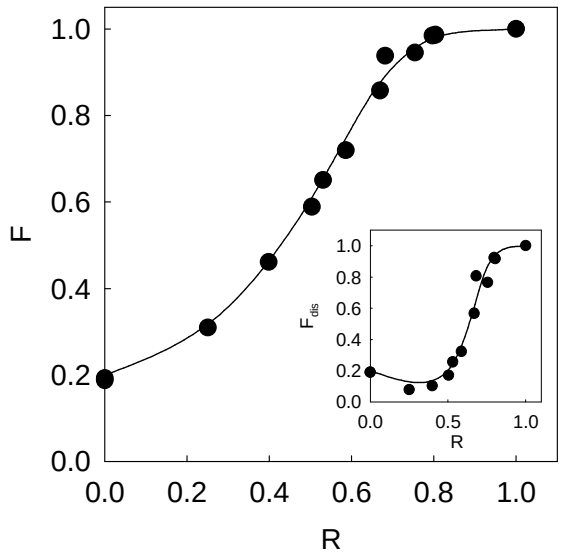

Figure 4. Fraction of non-sedimentable casein in mixtures containing a fixed NPCP concentration of 15g.L^{-1} as a function of the weight fraction of NaCas (R). The insert shows the fraction of dissociated casein micelles assuming that all added NaCas remained in the supernatant.

We have calculated the fraction of casein micelles that dissociated (F_{dis}) as a function of R assuming that added NaCas did not sediment. The results are shown in the insert of Figure 4.

Remarkably, when low weight fractions of NaCas were added F_{dis} decreased from 0.2 to 0.1 at R=0.25. This finding could not be explained by experimental uncertainty and implies that when a small amount of NaCas is added a fraction of the caseinate binds to the casein micelles. RP-HPLC chromatographs of the remaining NaCas in the supernatant showed that is mainly α_{s1}-casein that binds to the micelles, see below. At larger R, F_{dis} increased weakly up till R=0.5 followed by a sharp increase until almost all casein micelles were dissociated for R>0.75.

RP-HPLC chromatographs of the casein in the supernatant at different R are shown in Figure 5. As might be expected, the relative fraction of α_s-casein increased and the relative fractions of κ-casein and degraded caseins decreased with increasing R until we recovered the composition of the casein powders at R>0.75. Figure 6 shows the relative proportion of α_{s1}-, β- and κ-casein in the supernatant as a function R. The proportion of these three main casein components was almost the same in the NaCas and NPCP powders. At R=0.25 the relative fraction of α_{s1}-casein had not increased significantly and up to R=0.6 the proportion of α_{s1}-casein in the supernatant remained lower and that of κ-casein higher than in the powders. We speculate that at small R some of the α_{s1}-casein of the added NaCas was incorporated into the micelles and that this is the reason why F_{dis} as a function of R, shown in set of Figure 4, decreased at low R.

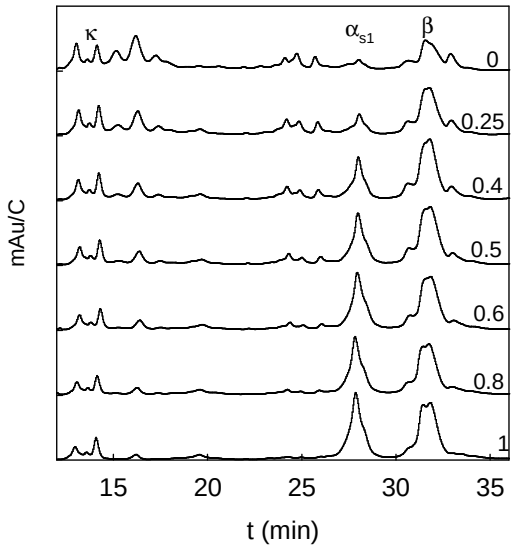

Figure 5. RP-HPLC chromatograms of the supernatant of mixtures containing 15g.L^{-1} NPCP and different weight fractions of NaCas as indicated in the figure.

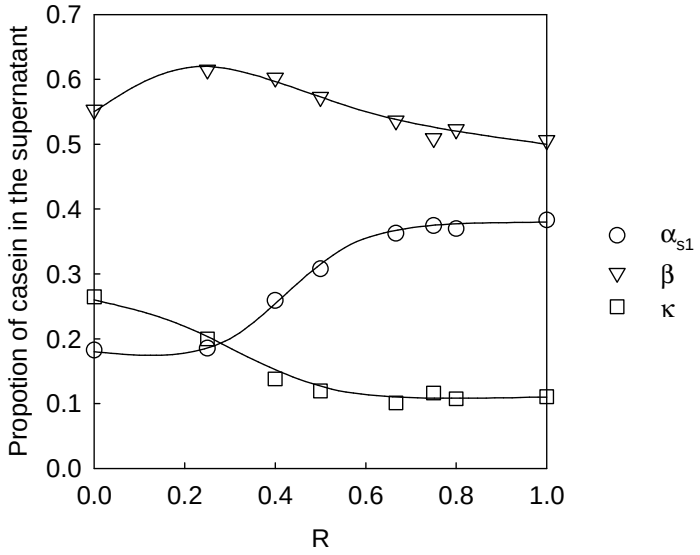

Figure 6. Proportion of the three main types of casein in the supernatant of mixtures containing 15g.L^{-1} NPCP as a function of the weight fraction of NaCas.

The amounts of calcium and phosphorus in the supernatant were analyzed as described in the materials and methods section. In order to calculate the fraction of inorganic phosphate that was released by dissociation of the casein micelles, the contribution of organic phosphorus from the phosphoserine groups was subtracted from the total phosphorus content. We considered that the concentration of organic phosphate in the micelles was the same as that of NaCas. This assumption is reasonable since the amount of inorganic phosphate in the NaCas sample was negligible. The results were normalized by the total amount of calcium and inorganic phosphate in the system and are shown in Figure 7 as a function of R.

The supernatant contained a significant fraction of calcium and phosphate (25%) even in the absence of added NaCas. The fraction remained approximately constant up to R=0.4 and increased steeply for R>0.6, i.e when NaCas was added in excess. The variation of the fraction of calcium and phosphate with R was approximately the same and the increase for R>0.6 was close to that of the fraction of dissociated casein micelles shown in Figure 4. However, we did not observe the decrease at small R that was observed for F_{dis}. The fraction of free calcium ions in the supernatant was negligible and preliminary results of ^{31}P NMR spectroscopy showed that the fraction of free orthophosphate was also very small. The implication is that calcium phosphate in the supernatant was bound to caseins, possibly in the form of nanoclusters.

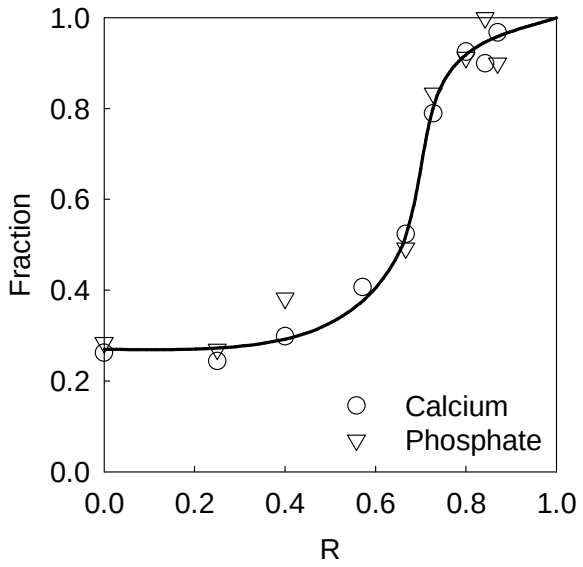

Figure 7. Fraction of calcium and phosphorus in the supernatant of mixtures containing 15g.L^{-1} NPCP as a function of the weight fraction of NaCas.

3.2.2. Turbidity

The dissociation kinetics of the casein micelles after mixing with NaCas was studied by measuring the turbidity as a function of time starting immediately after mixing. Figure 8 shows the evolution of the turbidity at τ=685nm for solutions containing a fixed NPCP concentration of 15g.L^{-1} and different amounts of NaCas. As was discussed above, the slow decrease of τ for the casein micelles at R=0 was due to spontaneous dissociation of the micelles in pure water. For R=0.21 a slight increase was observed followed by a very slow decrease as pure water. For R=0.51 the turbidity measured immediately after mixing was significantly lower and decreased very slowly at a rate comparable to that in pure water. At larger R, the initial drop of the turbidity increased and the rate at which is continued to decrease increased. A similar initial drop of the turbidity followed by slow decrease was reported by Pitkowski, et al. (2007) for casein micelle solutions after addition of EDTA or polyphosphate. We will compare the effect of adding NaCas with that of adding polyphosphate below.

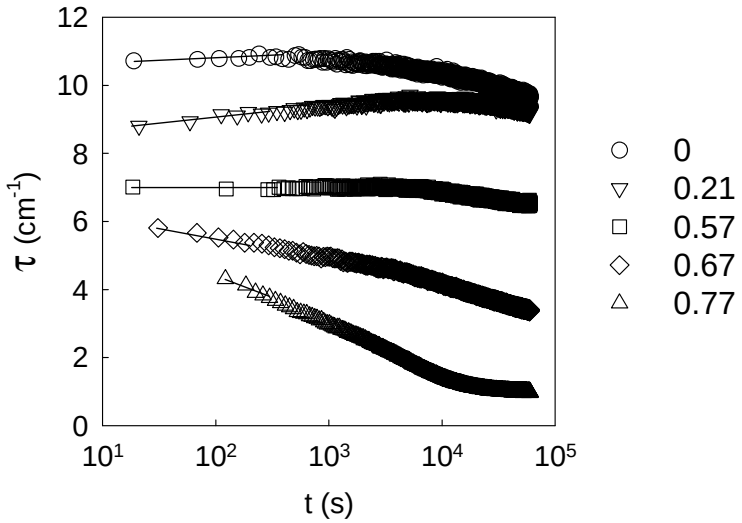

Figure 8.Turbidity at 685nm as a function of time for mixtures containing 15 g.L^{-1} NPCP and different weight fractions of NaCas as indicated in the figure.

Similar behavior was observed for solutions containing 5 g.L^{-1} or 10 g.L^{-1} NPCP. The turbidity normalized by the NPCP concentration is shown in Figure 9 as a function of R for three different NPCP concentrations. Results obtained shortly after mixing (1 min) are shown in Figure 9a while the results obtained 16h after mixing are shown in Figure 9b. In this representation the results obtained at different casein micelle concentrations are approximately the same indicating that the dissociation process depends on the ratio between casein micelles and NaCas and not on the total protein concentration. Addition of NaCas up to a fraction of about 40% did not result in a significant change of the turbidity apart from a weak maximum at R=2.5 in Figure 9b that corresponds to the weak minimum in F_{dis} shown in Figure 4. The decrease of the turbidity for R>0.5 was sharper after 16h (Figure 9b) than immediately after mixing as time was given for dissolution of the casein micelles via the second slow process. Notice, however, that for R<0.65 the system was still far from steady state after 16 h.

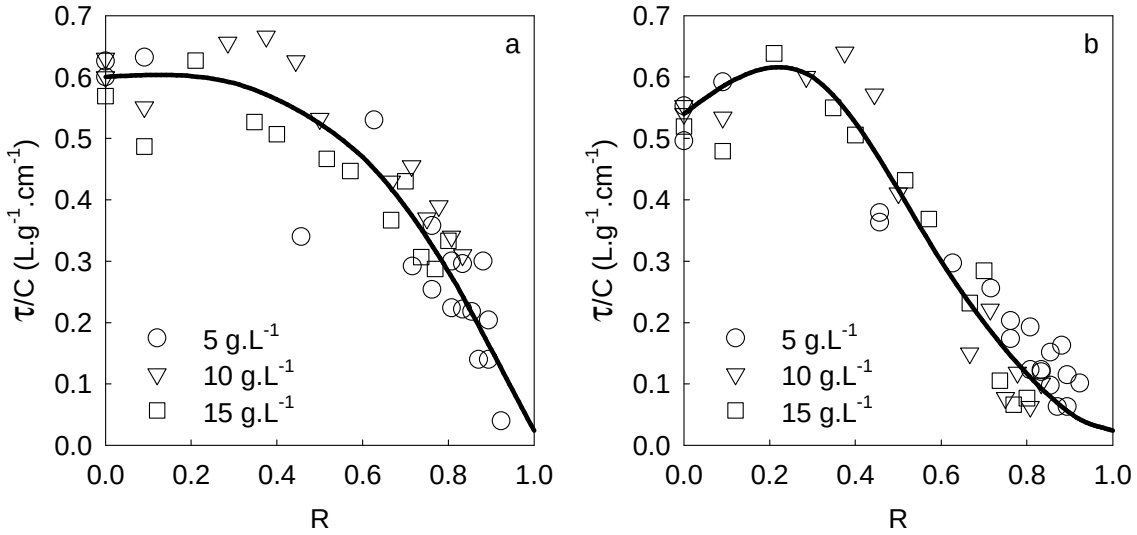

Figure 9.Turbidity at 685nm of mixtures containing different concentrations of NPCP as a function of the weight fraction of NaCas. The turbidity was measured 1min (a) and 16h (b) after preparation and was normalized by the NPCP concentration as indicated in the figure.

Assuming that the structure and size of the residual casein micelles in mixtures with NaCas is independent of R, the normalized turbidity is proportional to the fraction of intact micelles. In this case the variation of the turbidity should be comparable to the fraction of insoluble casein if all and only intact casein micelles sediment during centrifugation. In Figure 10 the fraction of intact casein micelles (F_{int}) calculated from the turbidity is compared to the fraction of insoluble casein as a function of the weight fraction of NaCas for mixtures containing 15g.L^{-1} NPCP 16h after mixing. In the calculation of the fraction of intact casein micelles we have taken into account that 20% of the casein micelles was dissociated at R=0. The results of the two independent measurements are the same within the experimental error confirming that the structure of the residual casein micelles does not depend strongly on R.

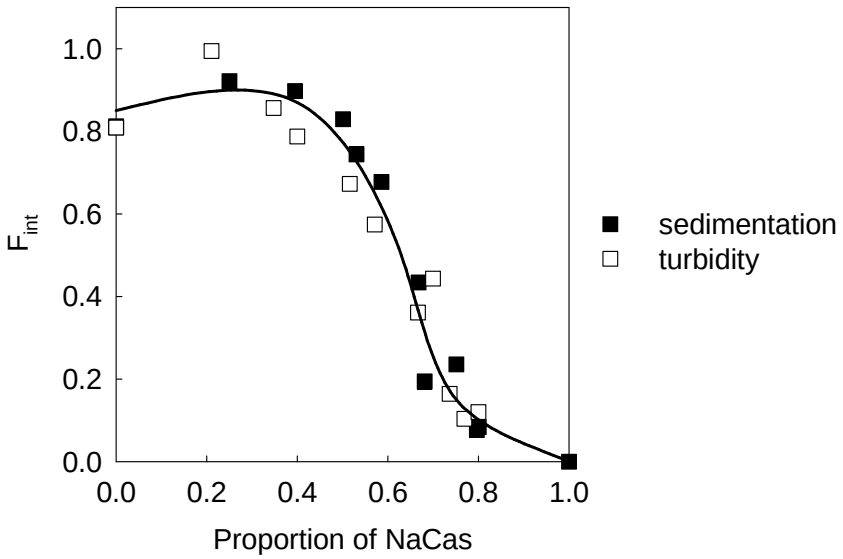

Figure 10. Fraction of intact casein micelles (Fint) in mixtures containing 15g.L^{-1} NPCP as a function of the weight fraction of NaCas calculated from turbidity measurements (open symbols) and centrifugation (closed symbols). The results were obtained 16h after preparation of the mixtures.

3.2.3. Aggregation

The long time evolution of the visual appearance of mixtures containing 15g.L^{-1} NPCP and different fractions of NaCas is shown in Figure 11. In pure water (R=0) the turbidity slowly decreased over a period of weeks. As we discussed above, the turbidity of mixtures one day after preparation decreased with increasing fraction of NaCas. Remarkably, however, after the turbidity had reached a minimum it increased again very slowly.

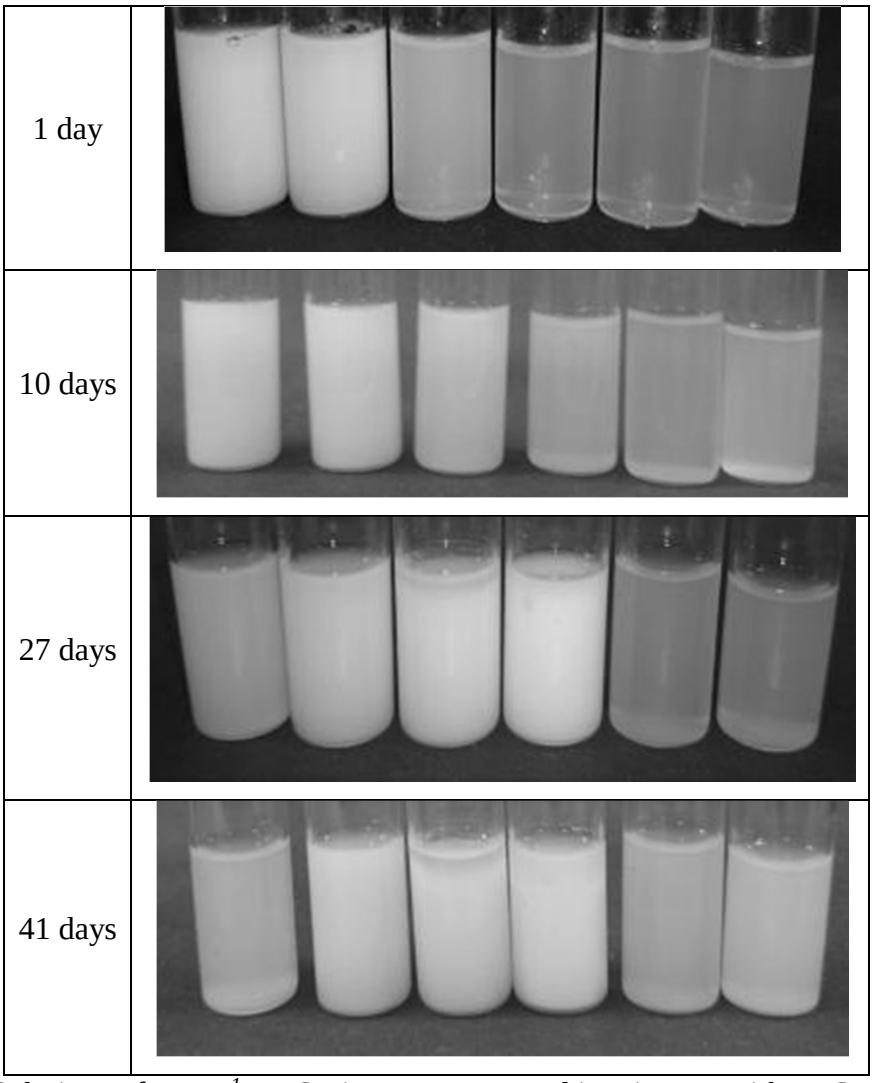

Figure 11. Solutions of 15g.L^{-1} NPCP in pure water and in mixtures with NaCas at different weight fractions at different times after preparation. R increased from left to right: 0, 0.5, 0.65, 0.75, 0.8 and 0.86.

The increase of the turbidity in the mixtures is most likely caused by aggregation of the caseins induced by the calcium phosphate that was released from the casein micelles. The rate of this process increased with increasing NPCP concentration and could at higher concentrations be seen in the evolution of the turbidity within the first day after mixing. This is illustrated for systems containing 20g.L^{-1} NPCP in Figure 12. In pure water the turbidity remained almost constant for the duration of the experiment (16h). The turbidity of mixtures immediately after preparation varied little up to R=0.5 and decreased at larger R, in accordance with the results at

lower NPCP concentrations shown above. However, at 20g.L^{-1} NPCP the turbidity increased slowly at longer times due to aggregation of caseins induced by the released calcium phosphate. The increase of the turbidity at longer times appears to become faster with increasing fraction of NaCas.

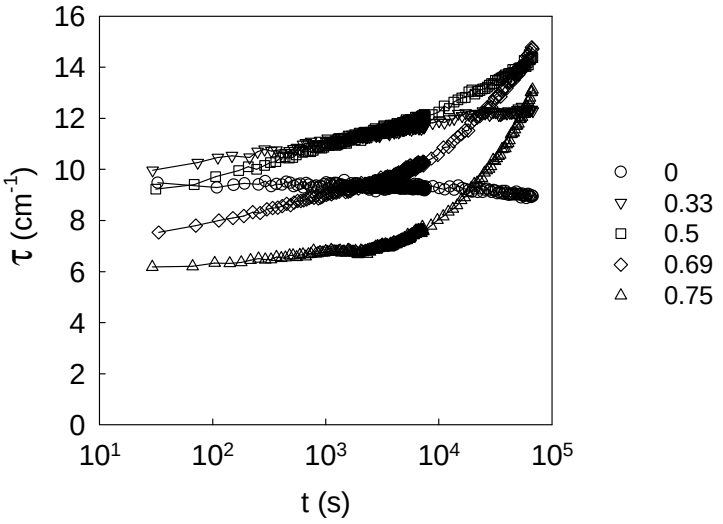

Figure 12. Turbidity at 685nm as a function of time for mixtures containing 20 g.L^{-1} NPCP and different weight fractions of NaCas indicated in the figure.

4. Discussion

The results presented here show that casein micelles dissociate when they are mixed with NaCas in aqueous solution. The added NaCas has a strong affinity for calcium and therefore calcium ions can migrate from the micelles to the soluble caseins reducing the CCP content in the former. The process of dissociation of the casein micelles after adding NaCas is similar to that observed after adding sodium polyphosphates or EDTA (Pitkowski, et al., 2007). In each case, dissociation of the micelles occurred in two stages : rapid dissociation of the casein micelles during or very shortly after mixing followed by slow progressive dissociation. The latter process occurred also in pure water, but its rate increased sharply with increasing concentration of added NaCas as was also reported elsewhere to occur when polyphosphate or EDTA are added (Pitkowski, et al., 2007). Dissociation of the micelles induced by polyphosphate or EDTA was shown to be an 'all or nothing process', during which the micelles either completely dissociate or remains largely intact (Pitkowski, et al., 2007). The fact that calcium and phosphate were released at the same rate as at which the casein micelles were solubilized suggests that

dissociation induced by NaCas also occurs in this manner. In addition, the reduction of the turbidity was close to the reduction of sedimentable casein, which also points to an 'all or nothing process'.

It is of interest to compare quantitatively the capacity of polyphosphate to induce dissociation of casein micelles with that of NaCas at the same pH and temperature, because for both systems calcium ions are chelated by phosphate groups. We will focus the comparison on the initial dissociation that occurred shortly after mixing that is not influenced by long time processes. It was found that the fraction of dissociated micelles decreased approximately linearly with increasing mass ratio of polyphosphate to NPCP. In order to compare these results with the present findings we need to express them in terms of the number of phosphate groups per gram of NPCP. In the calculation we have used the fact that NaCas and polyphosphate contained 0.25 mmol/g and 11 mmol/g phosphate groups, respectively. Figure 13 shows the fraction of intact micelles (F_{int}) remaining after dissociation immediately after mixing as a function of the number of added phosphate groups per gram of NPCP. As we mentioned above, in this representation the results do not depend on the NPCP concentration. The dashed line in the figure represents the results obtained with polyphosphate. The results obtained for the two systems are remarkably close implying that the capacity of phosphate groups to dissociate casein micelles is only weakly larger in polyphosphate than in sodium caseinate.

Recently, Silva, et al., (2013) studied the effect of removing a fraction of CCP from casein micelles by reducing the pH and subsequently dialyzing against milk ultrafiltrate. They observed that a fraction of the casein micelles was dissociated, but that the properties of the remaining micelles were close to that of native micelles. The fraction of dissociated micelles was increased in proportion with the amount of removed CCP. The composition of these solutions is comparable to that of mixtures of casein micelles and NaCas except that the content of free ions was kept constant and equal to that of the milk filtrate. The presence of minerals in the mixtures may influence their behavior and will need to be investigated in the future.

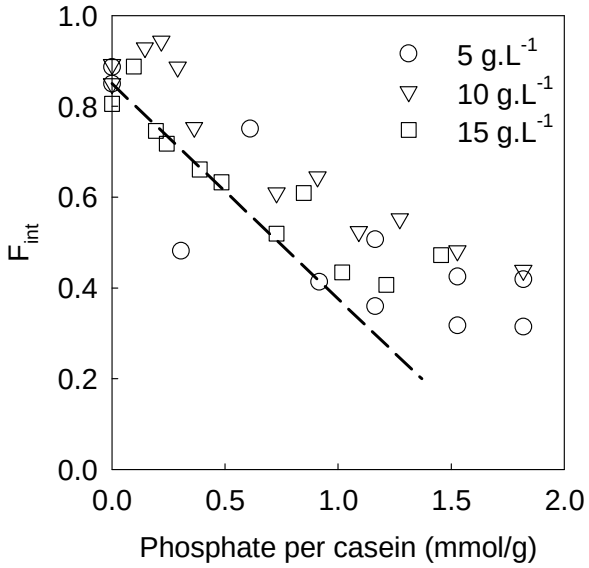

Figure 13. Fraction of intact casein micelles (F_{int}) in mixtures with different NPCP concentrations, as indicated in the figure, as a function of the number of phosphate units originating from added NaCas per gram NPCP. The dashed line indicates the fraction of intact casein micelles as a function of the number of phosphate units per gram NPCP originating from added polyphosphates (results taken from (Pitkowski, et al., 2007)).

Dissociation of casein micelles in the presence of NaCas was followed by aggregation of casein at longer times. It is clear that the effect of adding NaCas is not simply to chelate calcium ions as in the case of EDTA. It is well known that addition of calcium ions can cause aggregation of caseins (Pitkowski, et al., 2009; Thomar, et al., 2012; Zittle, et al., 1958). However, the added NaCas probably also binds the released orthophosphate together with the calcium ions, which may influence the aggregation process.

5. Conclusions

Addition of NaCas to aqueous casein micelle solutions at pH 6.7 leads to dissociation of the micelles following an 'all or nothing' process, comparable to that induced by calcium chelating agents such as EDTA and polyphosphate. Dissociation causes a strong decrease of the turbidity of casein micelles solutions when NaCas is added. The degree of dissociation increases rapidly when the weight fraction of NaCas in the mixtures exceeded 60% and is almost complete at 80%. In pure water and at low NaCas fractions κ-casein is preferentially solubilized while most

of the α_s-casein remains bound to the casein micelles. The calcium and orthophosphate ions that are released from the dissociated micelles bind to the caseins in solution and can induce slow aggregation of the casein.

References

Alvarez, E. M., Risso, P. H., Canales, M. A. M., Pires, M. S., & Gatti, C. A. (2008). Hydrodynamic properties-structure relationship for sodium caseinates in presence of calcium. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *327*(1-3), 51-56.

Aoki, T., Yamada, N., Kako, Y., & Imamura, T. (1988). Dissociation during dialysis of casein aggregates cross-linked by colloidal calcium phosphate in bovine casein micelles. *Journal of Dairy Research, 55,* 180-195.

Chu, B., Zhou, Z., Wu, G., & Farrell, H. M. J. (1995). Laser light scattering of model casein solutions: effects of high temperature. *Journal of Colloid and Interface Science, 170*(1), 102-112.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter, 7*(6), 2265-2272.

Dalgleish, D. G., & Parker, T. G. (1980). Binding of calcium ions to bovine α_{s1}-casein and precipitability of the protein-calcium ion complexes. *Journal of Dairy Research, 47,* 113-122.

Dalgleish, D. G., Paterson, E., & Horne, D. S. (1981). Kinetics of aggregation of α_{s1}-casein / Ca^{2+} mixtures: charge and temperature effects. *Biophysical Chemistry, 13*, 307-314.

de Kort, E., Minor, M., Snoeren, T., van Hooijdonk, T., & van der Linden, E. (2011). Effect of calcium chelators on physical changes in casein micelles in concentrated micellar casein solutions. *International Dairy Journal, 21*(12), 907-913.

De Kruif, C., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry, Vol. 1, Proteins. 3rd ed* (pp. 233-276). New York, NY, USA: Kluwer Academic/Plenum Publishers.

Farrell, H. M., Cooke, P. H., King, G., Hoagland, P. D., Groves, M. L., Kumosinski, T. F., & Chu, B. (1996). Particle sizes of casein submicelles and purified kappa-casein. Comparisons of

dynamic light scattering and electron microscopy with predictive three-dimensional molecular models. *A.C.S. Symposium Series, 650*, 61-79.

Farrell, H. M., Malin, E. L., Brown, E. M., & Qi, P. X. (2006). Casein micelle structure: what can be learned from milk synthetis and structural biology, *Current Opinion in Colloid & Interface Science,1*(2-3). 135-147.

Farrell Jr, H. M., Kumosinski, T. F., Pulaski, P., & Thompson, M. P. (1988). Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization. *Archives of Biochemistry and Biophysics, 265*, 146-158.

Farrer, D., & Lips, A. (1999). On the self-assembly of sodium caseinate. *International Dairy Journal, 9*, 281-286.

Gaygadzhiev, Z., Massel, V., Alexander, M., & Corredig, M. (2012). Addition of sodium caseinate to skim milk inhibits rennet-induced aggregation of casein micelles. *Food Hydrocolloids, 26*(2), 405-411.

Griffin, M. C. A., Lyster, R. L. J., & Price, J. C. (1988). The disaggregation of calcium-depleted casein micelles. *European Journal of Biochemistry, 174*, 339-343.

Guo, M., Campbell, B. E., Chen, K., Lenhoff, A. M., & Velev, O. D. (2003). Casein precipitation equilibria in the presence of calciums ions and phosphates. *Colloids and Surfaces B: Biointerfaces, 29*, 297-307.

HadjSadok, A., Pitkowski, A., Benyahia, L., Nicolai, T., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids, 22* 1460-1466.

Hansen, S., Bauer, R., Lomholt, S. B., Quist, K. B., Pedersen, J. S., & Mortensen, K. (1996). Structure of casein micelles studied by small-angle neutron scattering. *European Journal of Biochemistry, 24*, 143-147.

Holt, C. (1982). Inorganic constituents of milk III. *Journal of Dairy Research, 49*, 29-38.

Holt, C. (1992). Structure and stability of bovine casein micelles. *Advances of protein chemistry, 43*, 63-151.

Holt, C. (1998). Casein structure and casein-calcium phosphate interactions II. Dairy Science and Technology. In S. B. A/S (Ed.), *Proceedings of the 25th International Dairy Congress, Aarhus, 21-24 September 1998* (pp. 200-208): The Danish National Committee of the IDF.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion in Colloid & Interface Science, 11*(2), 148-153.

Kaliappan, S., & Lucey, J. (2011). Influence of mixtures of calcium-chelating salts on the physicochemical properties of casein micelles. *Journal of Dairy Science, 94*(9), 4255-4263.

Lin, S., Leong, S., Dewan, R., Bloomfield, V., & Morr, C. (1972). Effect of calcium ion on the structure of native bovine casein micelles. *Biochemistry, 11*(10), 1818-1821.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Food Chemistry, 48*, 1610-1616.

Marchin, S., Putaux, J.-L., Pignon, F., & Léonil, J. (2007). Effects of the environmental factors on the casein micelle structure studied by cryo-transmission electron microscopy and small angle x-ray scattering/ultra small-angle x-ray scattering. *Journal of Chemical Physics, 126*, 126-135.

McMahon, D. J., & Brown, R. J. (1984). Composition, structure and integrity of casein micelles: a review. *Journal of Dairy Science, 67*, 499-512.

Miranda, G., Mahé, M. F., Leroux, C., & Martin, P. (2004). Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics, 4(*8), 2496-2509.

Mizuno, R., & Lucey, J. (2005). Effects of emulsifying salts on the turbidity and calcium-phosphate–protein interactions in casein micelles. *Journal of Dairy Science, 88(*9), 3070-3078.

Morris, G. A. (2002). The self-assembly and structure of caseins in solution. *Biotechnology and Genetic Engineering Reviews, 19*, 357-376.

Mulvihill, D. M., & Fox, P. F. (1989). Caseins and caseinate manufacture. In P. F. Fox (Ed.), *Developments in Dairy Chemistry* (Vol. 4, pp. 97-130). London, UK: Elsevier Applied Science.

Nair, P. K., & Corredig, M. (2015). Rennet-induced gelation of concentrated milk in the presence of sodium caseinate: Differences between milk concentration using ultrafiltration and osmotic stressing. *Journal of Dairy Science, 98*(1), 1-10.

Panouillé, M., Durand, D., Nicolai, T., Larquet, E., & Boisset, N. (2005). Aggregation and gelation of micellar casein particles. *Journal of Colloid and Interface Science*, 85-93.

Panouillé, M., Nicolai, T., & Durand, D. (2004). Heat induced aggregation and gelation of casein submicelles. *International Dairy Journal, 14*, 297-303.

Parker, T. G., & Dalgleish, D. G. (1981). Binding of calcium ions to bovine beta-casein. *Journal of Dairy Research, 48*, 71-76.

Pitkowski, A., Nicolai, T., & Durand, D. (2007). Scattering and turbidity study of the dissociation of casein by calcium chelation. *Biomacromolecules, 9(*1), 369-375.

Pitkowski, A., Nicolai, T., & Durand, D. (2009). Stability of caseinate solutions in the presence of calcium. *Food Hydrocolloids*, *23*(4), 1164-1168.

Schmidt, D. G. (1982). Association of caseins and casein micelle structure. In P.F.Fox (Ed.), *Developments in Dairy Chemistry* (Vol. I, pp. 61-86). London: Applied Science Publishers, Essex.

Schuck, P., Piot, M., Mejean, S., Le Graet, Y., Fauquant, J., Brulé, G., & Maubois, J. L. (1994). Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane. *Le Lait, 74*(5), 375-388.

Silva, N. N., Piot, M., de Carvalho, A. F., Violleau, F. d. r., Fameau, A.-L., & Gaucheron, F. d. r. (2013). pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties. *Food Hydrocolloids, 32*(2), 322-330.

Stothart, P. H., & Cebula, D. J. (1982). Small-angle neutron scattering study of bovine casein micelles and sub-micelles. *J. Mol. Biol., 160*, 391-395.

Thomar, P., Durand, D., Benyahia, L., & Nicolai, T. (2012). Slow dynamics and structure in jammed milk protein suspensions. *Faraday Discussions, 158*, 325-339.

Visser, S., Slangen, C. J., & Rollema, H. S. (1991). Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 548*, 361-370.

Zittle, C. A., & Dellamonica, E. S. (1958). Binding of calcium to casein: influence of pH and calcium and phosphate concentrations. *Archives of Biochemistry and Biophysics, 76*, 342-353.

Chapter 7. Synergetic effects of orthophosphate and calcium on complex formation of sodium caseinate.

Synergetic effects of orthophosphate and calcium on complex formation of sodium caseinate.

Peggy Thomar, Alberto Gonzalez-Jordan, Jens Dittmer, Taco Nicolai.

LUNAM Université du Maine, IMMM UMR-CNRS 6283, Polymères, Colloïdes et Interfaces, 72085 le Mans Cedex 9, France.

Email : Taco.Nicolai@univ-lemans.fr

Abstract.

The effect of orthophosphate (0-0.1M) on aqueous solutions of sodium caseinate (NaCas) (C=10-80 g/L) in the presence of calcium ions was studied using confocal microscopy, sedimentation and ^{31}P MAS NMR. In the presence of calcium ions (0-0.1M), orthophosphate was observed to bind to the caseins leading to the formation of complexes involving all types of casein. Above a critical molar calcium/protein ratio in the complex, large protein aggregates were formed that sedimented during centrifugation. The critical molar ratio increased from about 6 without added orthophosphate to about 10 in excess orthophosphate (more than 40mM for C=80 g/L). Excess orthophosphate precipitated in the form of calcium phosphate particles and competed with caseins for binding of calcium ions. NMR showed that binding of calcium ions led to immobilization of inorganic orthophosphate and organic phophoserines in solid nanoparticles. In excess $CaCl_2$ and orthophosphate the complexes contained about 13 calcium and 10 orthophosphate ions.

1. Introduction.

The major protein in milk is casein and consists of mainly four different types (α_{s1}-, α_{s2}-, β- and κ-casein) that are assembled into micelles with an average radius of about 100nm held together by colloidal calcium phosphate nanoclusters (CCP) [1-2]. Below pH 5.0 these so-called casein micelles precipitate and the CCP is dissolved. After washing, the proteins can be resolubilized by increasing the pH using NaOH to obtain sodium caseinate (NaCas). NaCas powders are extensively used for functional and nutritional properties in many food applications [3]. In aqueous solution, sodium caseinate is organized into small particles of 11nm containing approximately 15 casein molecules weakly depending on the NaCl concentration and the temperature [4-5].

α_s- and β- casein are well-known to specifically bind Ca^{2+} in aqueous solution which reduces electrostatic repulsion and induces aggregation [6-8] whereas κ-casein does not interact strongly with Ca^{2+}. Bound Ca^{2+} induces attractive interactions between the NaCas particles which leads above a critical concentration of Ca^{2+} to the formation of domains [9]. As a consequence, the turbidity increases and the solubility decreases [9-10]. The effect is stronger at higher temperatures[9], but can be mitigated by adding monovalent ions which can drive bound Ca^{2+} to solution when they are present in large excess [9].

Orthophosphate (P_i) is widely used as a calcium chelating agent in many dairy products [11] and affects the interaction between Ca^{2+} and caseins. By itself, P_i does not bind onto caseins and therefore does not change the structure of [12]. However, in combination with calcium ions, orthophosphate induces the formation of colloidal suspensions of caseinate which under particular conditions can have a size and structure close to that of native casein micelles [13-15]. Orthophosphate was found to play an active role in the calcium induced precipitation of α_{s1}-casein [16] and β-casein [17]. It also increases the fraction of sodium caseinate that

precipitates in the presence of calcium ions [17-19]. This phenomenon strongly depends on the pH and ionic strength [19-20] and was attributed to the formation of complexes between calcium, phosphate caseins [17-19].

It is obvious that the interaction between orthophosphate, calcium and casein is very important both for the behavior of native casein micelles and for the properties of many dairy products, but the interaction is complex and despite past efforts it is as yet far from being fully elucidated. Almost all past investigations of the interaction between calcium, phosphate and caseinate were done at low protein concentrations, whereas the concentration can be quite high in food products. As far as we are aware, no systematic studies as a function of the casein concentration including concentrated solutions have been reported.

Understanding of the interaction with calcium and phosphate at higher protein concentrations is important in order to texture dense casein suspensions in a controlled manner. Therefore we have done a systematic study of the interaction of these ions with NaCas over a wide range of mineral and protein concentrations. The mesoscopic structure was studied using confocal laser scanning microscopy (CLSM). The fraction of sedimentable casein and minerals was determined by centrifugation and the composition of the supernatant was analyzed by chromatography. The mobility of organic and inorganic phosphorus was determined by ^{31}P NMR MAS. This technique has already shown its usefulness to characterize dairy systems in the past [21-23], but has not yet been used to study the interaction between caseinate, calcium and orthophosphate.

2. Materials and Methods

2.1 Sample preparation. The NaCas powder provided by Armor Protéines (Saint-Brice, France) contained 90% (w/w) protein, 1.3 % sodium, 0.7% phosphorus and trace amounts of whey proteins (<1%). The phosphorus content is principally organic in the form of phosphoserines, but NMR showed that the powder also contained about 7% of the total phosphorus content in the form of inorganic orthophosphate. The casein composition was determined by HPLC as 37, 4, 46 and 13% of α_{s1}-, α_{s2}-, β- and κ-caseins, respectively.

Initial solutions of NaCas were prepared by dispersing the powder in milliQ water (Millipore), containing 3mM sodium azide in order to avoid bacterial proliferation. The dispersions were heated at 80°C during 30 minutes, which led to complete solubilization of the casein. The solutions were centrifuged at $5\times10^4 g$ for 2h at 20°C in order to remove a small weight fraction of phospholipid/protein particles (Pitkowski et al., 2009). A top layer composed of residual fat and a small weight fraction of proteins (<5%) were delicately removed and clear supernatants were collected. The required concentrations of salts were obtained by slow addition of aliquots of 0.5M $CaCl_2$ or 0.5M sodium orthophosphate (Na_3PO_4/NaH_2PO_4) solutions at 20°C. The pH was adjusted at pH 6.7 by slow addition of aliquots of 0.1-1M HCl or NaOH under continuous stirring. The protein concentration (C) was determined by UV absorption at 280nm using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France) assuming an extinction coefficient of 0.85 $L.g^{-1}.cm^{-1}$. The protein concentration was set by adding water to more concentrated stock solutions. The pH became stable within one hour after preparation, but the solutions were left overnight after preparation before use.

2.2 RP-HPLC. The composition of the caseins was determined using Reverse Phase High Pressure Liquid Chromatography (Ultimate 3000, Dionex) with an analytical column C5 Jupiter

(250 x 4.6 mm), 30nm pore size, 5µm particle size (Phenomenex, Torrance, USA). The measurements were done as described elsewhere [24], and the protein detection was done by UV absorption at 214 nm and 25°C.

2.3. Mineral content determination. The calcium concentration in the NaCas solutions was obtained by atomic flame spectrometry at 422.7nm (Varian AA240FS, Les Ulis, France) and the total phosphorus concentration in the solutions was determined using the method reported in [25]. The fraction of calcium ions in the supernatant (F_{Ca}) was determined by normalizing the concentration in the supernatant with the total concentration in the solution. The fraction of bound Ca^{2+} was determined by measuring the calcium ion activity using a calcium-selective electrode (Fisher Scientific, Hampton, NH, USA), which was calibrated by measuring the potential of aqueous solutions of $CaCl_2$ as a function of the concentration between 1 and 50mM. The activity of Ca^{2+} that was bound to the proteins was assumed to be negligible.

2.4 Confocal Laser Scanning Microscopy (CLSM). CLSM observations were made using a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany) as described in [9].

2.5 ^{31}P NMR. The NMR experiments were conducted on a Bruker Avance III 300 MHz WB spectrometer equipped with a 4 mm MAS VTN type probe head with two channels. An HR/MAS rotor was filled with ≈55 µL of the NaCas solution and spun at 3 kHz as reported in [21].

2.6 Centrifugation. Solutions were centrifuged at room temperature using an Allegra 64R centrifuge (Beckman Coulter, USA) at 5×10^4g.

3. Results

3.1. Mesoscopic structure

Our objective was to study the interaction of caseinate with calcium and phosphate as a function of the protein concentration and in particular at higher protein concentrations. Up to C=80 g/L it is still relatively easy to separate aggregated caseinate particles by centrifugation in order to determine their composition, but this becomes increasingly difficult at higher concentrations, because the viscosity increases sharply. Therefore we studied in first instance solutions of NaCas at 80g/L containing a wide range of $CaCl_2$ (0-80mM) and orthophosphate (P_i) (0-100mM) concentrations. The pH was fixed at 6.7.

CLSM images of the different structures that were formed are shown in Figure 1. As was reported in [9], increasing the molar concentration of $CaCl_2$ ([$CaCl_2$]) in the absence of P_i leads to formation of dense protein aggregates possibly induced by microphase separation. Here we find that addition of 20-50mM P_i inhibited formation of these aggregates and more $CaCl_2$ was needed to induce their formation. Large excess of P_i (>50mM) led to the formation of calcium phosphate particles covered by a layer of adsorbed proteins as was earlier reported in [17].

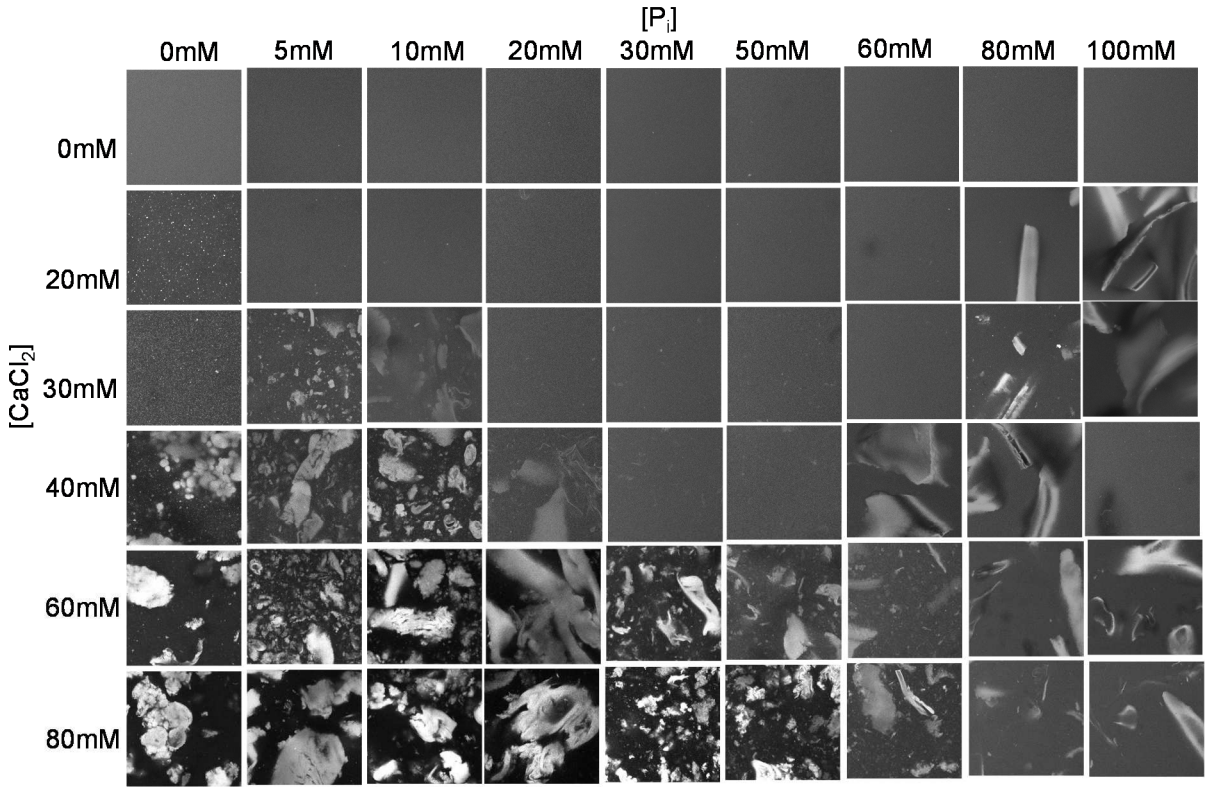

Figure1. CLSM images (159µm × 159µm) of aqueous solutions of 80g/L NaCas at different calcium and orthophosphate concentrations.

3.2 Fraction of aggregated NaCas particles

3.2.1 Effect of the orthophosphate concentration.

In order to determine the fraction of caseinate that associated into large aggregates by complexation with calcium and phosphate, the caseinate solutions were centrifuged at $5\times10^4 g$ for 2h at 20°C. At this centrifugation rate the NaCas particles with a radius of about 10nm that are formed in water or at low calcium concentrations [26] do not sediment, but all large aggregates formed with calcium and phosphate do sediment. The fraction of protein in the supernatant (F_p) was determined by measuring the UV absorbance as explained in the materials and methods section.

F_p is plotted in Figure 2a as a function of [$CaCl_2$] for different concentrations of P_i. Up to [$CaCl_2$]=25mM no sedimentation of protein was observed independent of the P_i concentration. At 5 or 10mM P_i, the fraction of protein in the supernatant decreased rapidly for [$CaCl_2$]>25mM. At these low orthophosphate concentrations, the critical amount of $CaCl_2$ needed to induce sedimentation of casein was the same as in the absence of P_i, but the decrease of F_p was steeper. In addition, for [$CaCl_2$]>40mM, nearly all caseins precipitated, whereas without P_i about 25% remained in the supernatant. A similar result was reported [17] who studied caseinate at C=5g/L at pH 7.5 and compared sedimentation induced by Ca^{2+} with and without orthophosphate. We will show below that the 25% casein remaining in the supernatant consisted of the κ-casein and about half of the β-casein.

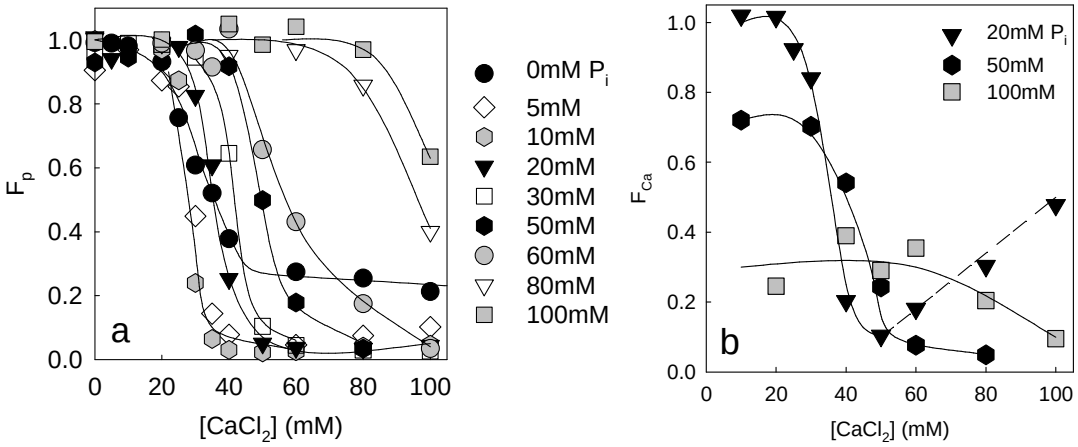

Figure 2a. Fraction of protein in the supernatant (F_p) for solutions of 80g/L NaCas plotted as a function of the Ca^{2+} concentration for different concentrations of P_i. The lines serve as guide to the eye.

Figure 2b. Fraction of calcium in the supernatant (F_{Ca}) of 80 g/L NaCas solutions as a function of the added $CaCl_2$ concentration at different concentrations of orthophosphate as indicated in the figure. The lines serve as guide to the eye.

It is remarkable that the critical $CaCl_2$ concentration above which casein started to sediment remained the same when 10mM P_i was present. Considering that P_i is integrated in the complex formation with casein and Ca^{2+} and therefore increases the net negative charge of the complex,

one might have expected an increase of the critical concentration. An increase of the critical $CaCl_2$ concentration with increasing P_i was in fact observed for $[P_i]$ >10mM. In all cases, the onset of the decrease of F_p corresponded to the appearance of large proteins aggregates that were visible in the CLSM images shown in Figure1.

Figure 2b shows the fraction of Ca^{2+} (F_{Ca}) that remained in the supernatant as a function of the total Ca^{2+} concentration for $[P_i]$=20 mM, 50 mM and 100 mM. At $[P_i]$=20 mM, the dependence of F_{Ca} on $[CaCl_2]$ was closely correlated to that of F_p for $[CaCl_2]$<50 mM. At this P_i concentration no calcium phosphate particles could be seen in the CLSM images. This suggests that calcium precipitated only in the form of complexes with NaCas and P_i. For $[CaCl_2]$>50 mM, Ca^{2+} was in excess and could not be fully incorporated in the protein/phosphate complexes, leading to an increase of F_{Ca} with increasing $[CaCl_2]$.

At $[P_i]$=100 mM, a large fraction of the calcium sedimented even for $[CaCl_2]$<80 mM where almost all proteins remained in the supernatant. CLSM showed that at these conditions calcium sedimented in the form of calcium phosphate particles, see Figure 1. The same phenomenon occurred to a lesser extent at $[P_i]$=50 mM for $[CaCl_2]$<40 mM. At higher $CaCl_2$ concentrations, F_p started to decrease due to sedimentation in the form of complexes with Ca^{2+} and P_i, which caused also a further decrease of F_{Ca}. Interestingly, if we subtract the amount of calcium that formed calcium phosphate particles from the total Ca^{2+} concentration in the system, we find that sedimentation of casein started when the residual Ca^{2+} concentration was approximately 30mM.

It is important to mention that for these experiments P_i was added to the NaCas solution before $CaCl_2$ was added. The order of mixing was not important when only casein/P_i/Ca^{2+} complexes were formed, but it was important at higher P_i concentrations at which calcium phosphate particles were formed. If first $CaCl_2$ was added, Ca^{2+} bound to the caseins inducing aggregation

and these aggregates did not disperse when P_i was added subsequently even at 100mM. However, if P_i was present in excess before $CaCl_2$ was added it competed for Ca^{2+} with the caseins leading to the formation of calcium phosphate particles.

3.2.2. Effect of the protein concentration

The dependence of F_p on [$CaCl_2$] was determined at different NaCas concentrations between 10 and 80 g/L for solutions without P_i and in the presence of 20mM, 30mM or 50mM P_i. The results are plotted in Figure 3 as a function of the molar ratio of Ca^{2+} to NaCas, i.e. the number of calcium ions per casein molecule, calculated as: $R=[CaCl_2].M/C$, where $M=2.3\times10^4$ g/mol is the average molar mass of the caseins. In this representation the results superimpose well for solutions without P_i and at [P_i]=20 mM, which means that aggregation of caseins is determined by the number of Ca^{2+} ions per casein. At [P_i]=30 mM and in particular at [P_i]=50 mM, we find that a significant fraction of the proteins remained in the supernatant at large values of R at lower protein concentrations. The reason is most likely that if less protein is present, P_i can compete more effectively for Ca^{2+} leading to the formation of calcium phosphate particles and a reduction of the amount of Ca^{2+} that binds to casein.

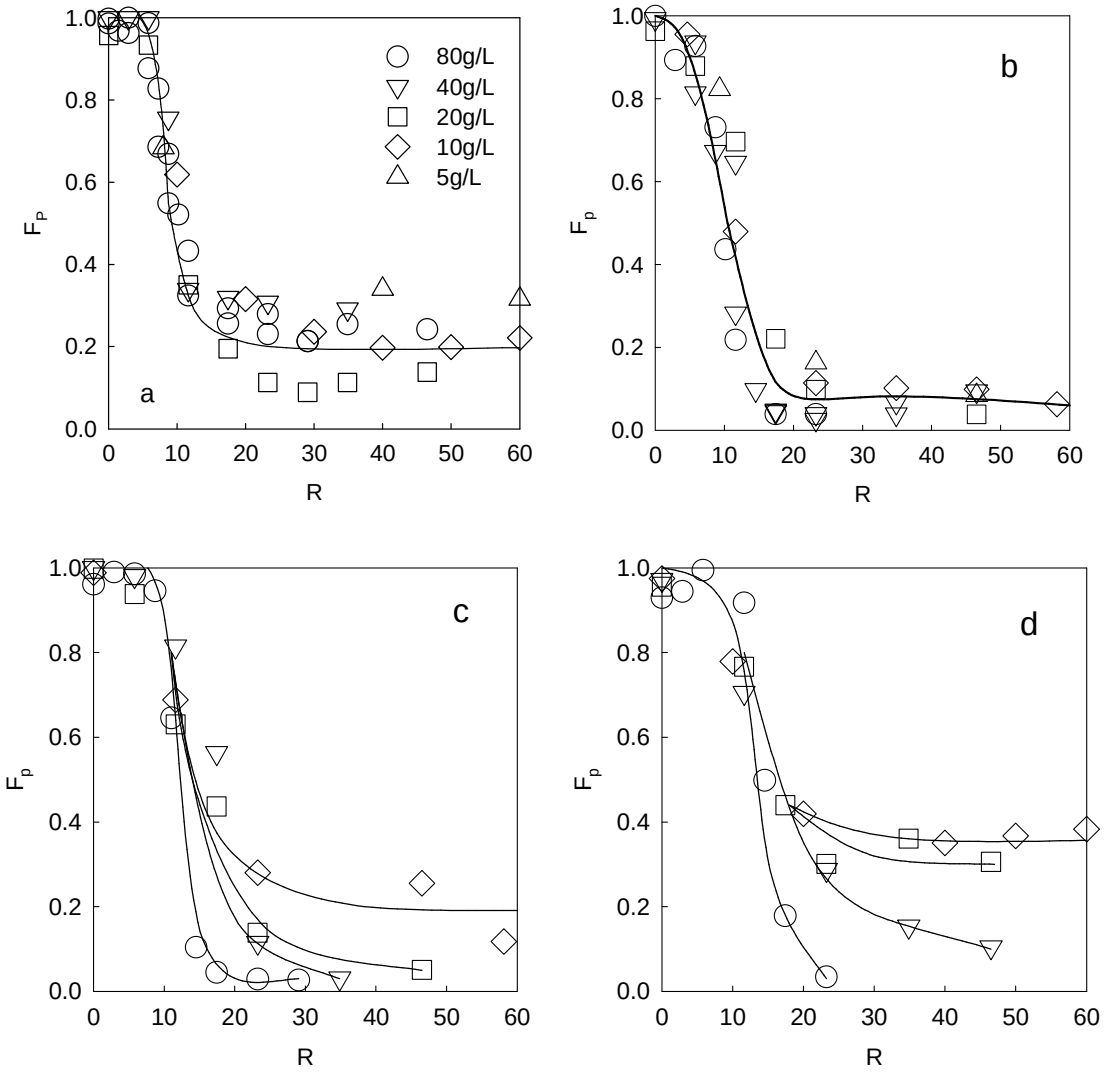

Figure 3. Fraction of proteins in the supernatant (F_p) of NaCas solutions as a function of R for different protein concentrations indicated in the Figure at [P_i]=0mM (a), 20mM (b), 30mM (c) or 50mM (d). The lines serve as guide to the eye.

3.2.3. Protein and mineral composition

A more detailed investigation of the effect of P_i was made for NaCas at 80 g/L at [P_i]= 20 mM. At this concentration F_p depends on R over the whole investigated [$CaCl_2$] range. The chromatograms of the supernatant obtained by RP-HPLC showed a series of peaks that could be attributed to the different caseins, see Figure 4. The composition of pure solutions NaCas determined in the absence of $CaCl_2$ contained 37, 4, 46 and 13% of α_{s1}-, α_{s2}-, β- and κ-casein, respectively.

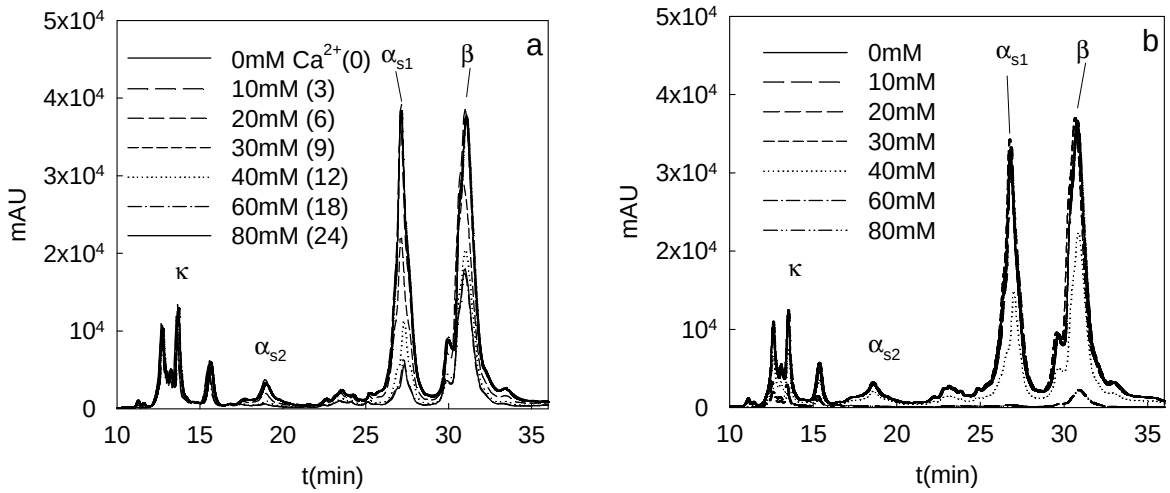

Figure 4. HPLC chromatograms of the soluble fraction of NaCas at 80 g/L for different concentrations of $CaCl_2$ without (a) and with 20mM P_i (b).

In the absence of P_i, the amount of α_s- and β- casein in the supernatant decreased with increasing [$CaCl_2$], whereas the κ-casein content remained almost constant. For [CaCl2]>50 mM (R>14) most of the α_s-caseins sedimented, but approximately half of the β-casein remained in the supernatant, see Figure 5. Interestingly, in the presence of phosphate the fraction in the supernatant of each type of casein depended on [$CaCl_2$] and the even κ-casein sedimented. The implication is that the well-known specific sensitivity to Ca^{2+} of the different types of casein is less important when complexes with P_i are formed.

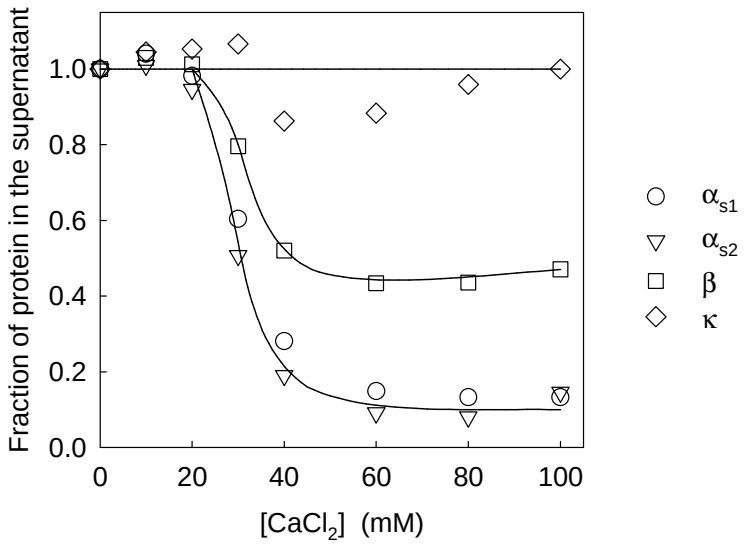

Figure 5. Fraction of the different types of casein in the supernatant (5×10^4g, 2h, 20°C) of sodium caseinate solutions at 80 g/L, pH 6.7, as a function of the calcium concentration. Lines drawn through the data are guides to the eye.

The total phosphorus concentration in the supernatant was determined as described in [25] and is shown as a function of the $CaCl_2$ concentration in Figure 6. In the absence of $CaCl_2$, all casein and phosphate is situated in the supernatant and the total phosphorus content was 41mM of which $[P_i]$=20 mM is inorganic from the added orthophosphate. The rest is principally organic phosphorus from the phosphoserines in the NaCas (P_o). For comparison we also show in Figure 6 the concentration of phosphorus in the supernatant for an aqueous solution of 20mM P_i as a function of the $CaCl_2$ concentration.

In the absence of NaCas the concentration of soluble P_i decreased linearly with the $CaCl_2$ concentration due to formation of solid calcium phosphate particles with a molar ratio of 1.6 Ca^{2+} per P_i in agreement with literature results [27-28]. In the presence of NaCas with or without added orthophosphate, all phosphorus remained in the supernatant for $[CaCl_2]\leq20$ mM and decreased sharply at higher concentrations. These results show that the added orthophosphate is bound to the caseins in the form of casein/Ca^{2+}/P_i complexes.

Calcium is present in three forms: I) as free ions, II) bound to casein in small aggregates that did not sediment and III) bound to casein in large aggregates that sedimented. The concentration of free Ca^{2+} ions in the supernatant was determined by measuring the activity of Ca^{2+} as was described in the materials and methods section. Figure 7a compares the concentration of free Ca^{2+} (I) and the total calcium concentration in the supernatant (I+II) as a function of $[CaCl_2]$ (I+II+III) for NaCas solutions without added P_i. For $[CaCl_2]<20$ mM no calcium sedimented, but the fraction of free Ca^{2+} in the supernatant was negligible implying that at these conditions all Ca^{2+} was bound to small casein aggregates. For $[CaCl_2]>20$ mM, the total concentration of

calcium in the supernatant was no longer proportional to [$CaCl_2$], because an increasing fraction of calcium sedimented together with the proteins. The fraction of free Ca^{2+} started to increase progressively for [$CaCl_2$]>20 mM and at [$CaCl_2$]>70 mM almost all the calcium in the supernatant was free, because all α-casein and half of the β-casein sedimented at these conditions. These results allow us to estimate that the total amount of Ca^{2+} that was bound to the proteins in excess $CaCl_2$ was about 30 mM, i.e. R≈8.

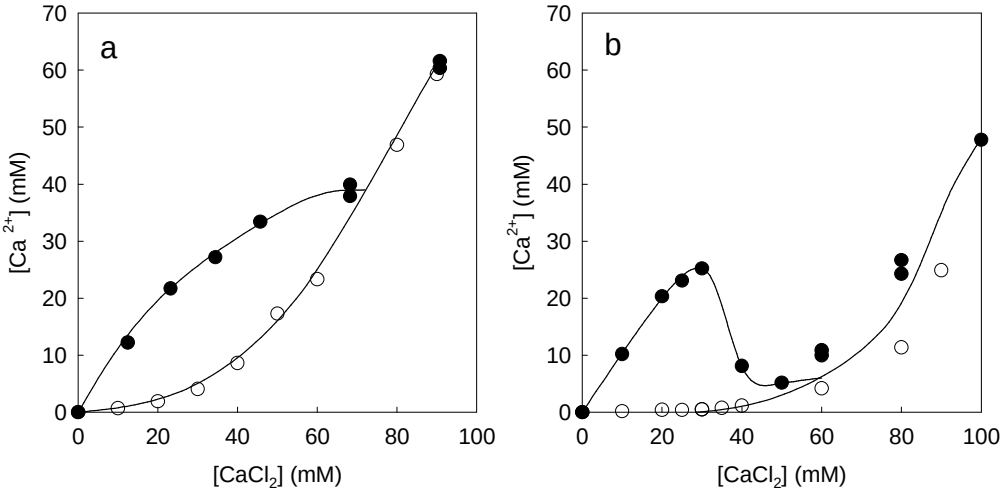

Figure 7. The total calcium concentration (filled symbols) and the concentration of free Ca^{2+} (open symbols) in the supernatant as a function of the added $CaCl_2$ concentration for solutions of NaCas at 80g/L without (a) or with (b) 20mM P_i. The lines serve as guide to the eye.

In the presence of 20 mM P_i, again no calcium sedimented for [$CaCl_2$]<20 mM. However, in the presence of orthophosphate the concentration of calcium in the supernatant (II) decreased sharply with increasing [$CaCl_2$] above 30 mM, whereas without P_i it continued to increase. Very little free Ca^{2+} was found for [$CaCl_2$]≤60 mM implying that more Ca^{2+} was bound when the complexes with casein included P_i. From the fraction of free Ca^{2+} at high [$CaCl_2$], we deduced

that the total amount of Ca^{2+} bound to the caseins was about 50 mM, i.e. R=13. This means that the presence of 20 mM P_i allowed the proteins to bind an extra 5 calcium ions per protein.

3.3. Mobility of organic and inorganic phosphate

^{31}P NMR experiments were done as a function of the $CaCl_2$ concentration for NaCas solutions at C=100 g/L containing 0, 20 or 50 mM orthophosphate, see Figure 8. As discussed in [21], the spectrum of pure NaCas solutions contains three peaks representing the signals of the phosphorus from the different phosphoserines (P_o) organized in clusters along the backbone. In addition, there is a sharp peak at 1.5 ppm due to a small fraction (7%) of inorganic orthophosphate (P_i) in the NaCas powder. For [$CaCl_2$]>10 mM, the amplitude of the phosphoserine signal decreased with increasing $CaCl_2$ concentration. The reason is that the ^{31}P NMR signal is no longer detected in the form of narrow peaks when the phosphorus is immobile. Therefore the decrease of the peak amplitude corresponds to an increasing fraction of immobile phosphoserines. The amplitude of the three peaks did not decrease in the same manner, because calcium binding is different for different phosphoserine clusters [1-2] and their immobilization with increasing [$CaCl_2$] is different. The signal of inorganic phosphate also decreased showing that it was incorporated into the calcium/casein complex.

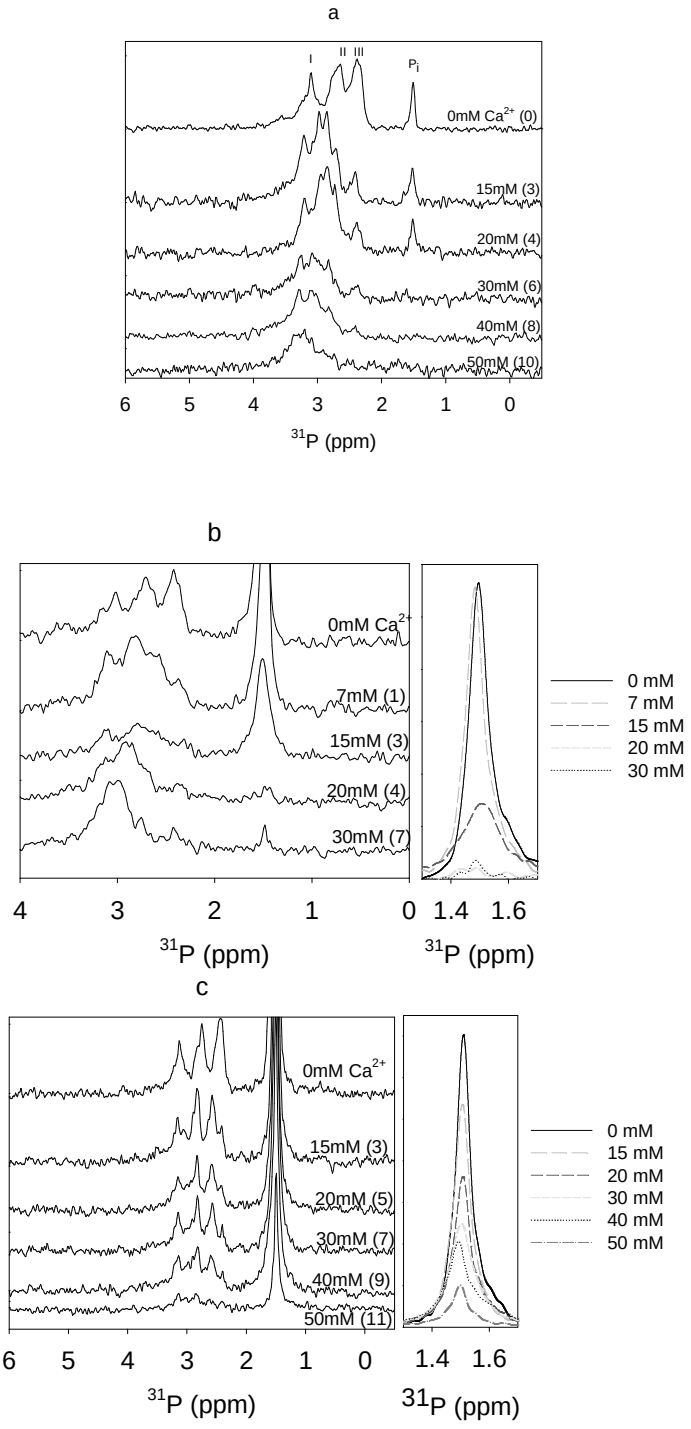

Figure 8. ^{31}P MAS NMR spectra of aqueous solutions of 100g/L NaCas at different $CaCl_2$ concentrations indicated in the figure. The solutions contained $[P_i]$=0mM (a), 20mM (b) or 50mM (c). The right panels show the P_i signal on an extended scale

The fraction of immobile phosphorus is plotted as a function of [$CaCl_2$] in Figure 9. It increased with increasing [$CaCl_2$] for [$CaCl_2$]>15 mM. Notice that even though for [$CaCl_2$]≤20 mM all proteins remained in the supernatant, see Figure 2, nevertheless a significant fraction of phosphoserines was immobile. This means that a fraction of the phosphoserines formed small solid clusters with Ca^{2+} without inducing large scale association of the caseinate particles. The latter is evident from the homogeneous CLSM images that showed that structure larger than hundred nm were formed, see Figure 1.

Addition of 20mM or 50mM orthophosphate, caused a large increase of the peak at 1.5ppm. The signal of both the organic phosphoserine and the inorganic orthophosphate decreased progressively with increasing $CaCl_2$. From the relative decrease of the signal amplitude the concentrations of immobile phosphoserine and orthophosphate were estimated, see Figure 9. Interestingly, it appears that within the experimental scatter the amount of immobilized phosphorus is determined by the amount of calcium independent of the amount of added orthophosphate. We note that for the systems explored with NMR the fraction of P_i that formed large calcium phosphate particles instead of nanoclusters was negligible. This suggests that the amount of P_i that is incorporated in complexes with casein and Ca^{2+} is equal to the amount of immobile P_i shown by the filled symbols in Figure 9 and is independent of the total amount of P_i. The ratio of $Ca^{2+}/P_i \approx 1.3$ in the complexes, which is somewhat smaller than what is reported for native casein micelles (1.5-2) but still within the experimental uncertainty.

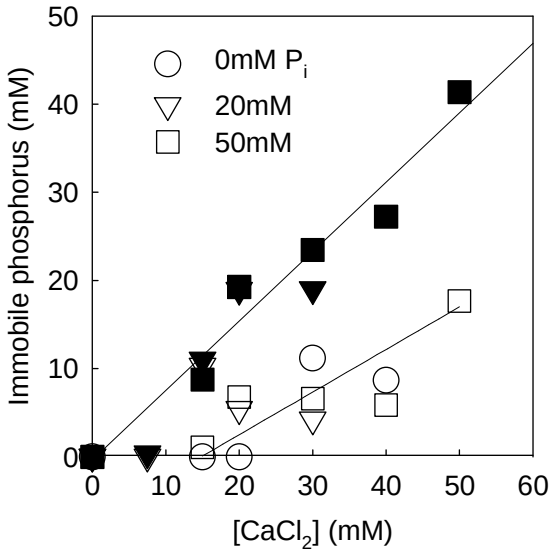

Figure 9. Concentration of immobile phosphoserine (open symbols) and inorganic phosphate (closed symbols) as a function of the $CaCl_2$ in solutions of NaCas at 100g/L. The straight lines represent fits to a linear dependence.

As was shown above that in the presence of orthophosphate 13 calcium ions are bound per protein when excess $CaCl_2$ is added. This means that in excess $CaCl_2$, the casein/Ca^{2+}/P_i complexes contain about 13 Ca^{2+} and 10 P_i in the form of solid calcium phosphate nanoparticles. These numbers may be compared to the composition of native casein micelles that contain about 17 Ca^{2+} and 9 P_i per casein [29].

4. Conclusions

When $CaCl_2$ is added to NaCas solutions Ca^{2+} binds specifically to the proteins, which leads for R>6 to large scale aggregation of almost all α-casein and 50% of the β-casein, but no κ-casein. Added orthophosphate is incorporated into the casein/Ca^{2+} complexes, which leads to large scale association involving all types of casein for R>10. When the molar ratio of

orthophosphate to casein is larger than about 10, it competes with casein for Ca^{2+} and forms microscopic calcium phosphate particles covered with protein.

NMR measurements showed that when Ca^{2+} binds to casein both organic and inorganic phosphorus becomes immobile implying that solid nanoparticles are formed that contain phosphoserines as well as added orthophosphate. In the absence of orthophosphate, caseins can bind on average up to 8 calcium ions per protein, but the presence of excess orthophosphate up to 13 Ca^{2+} are bound per protein. In excess $CaCl_2$ and orthophosphate, the casein/Ca^{2+}/P_i complexes contain on average about 10 P_i and 13 Ca^{2+} per casein.

References

1. Dalgleish, D.G., On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter*, 2011. 7(6): p. 2265-2272.

2. de Kruif, C.G. and C. Holt, Casein micelle structure, functions and interactions, in *Advanced Dairy Chemistry* Volume 1: Proteins, 3rd edn, K.A. P.F. Fox and P.L.H. McSweeney, Editor. 2003, Plenum Publishers.

3. Southward, C.R., Used of casein and caseinates, in *Developement in Dairy Chemistry-4*, F.P. Fox, Editor. 1989, Elsiever Applied Science: London. p. 173-244.

4. Chu, B., et al., Laser light scattering of model casein solutions: effects of high temperature. *Journal of Colloid and Interface Science*, 1995. 170(1): p. 102-112.

5. HadjSadok, A., et al., Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids*, 2008. 22 p. 1460-1466.

6. Dalgleish, D.G. and T.G. Parker, Binding of calcium ions to bovine α_{s1}-casein and precipitability of the protein-calcium ion complexes. *Journal of Dairy Research*, 1980. 47: p. 113-122.

7. Dalgleish, D.G., E. Paterson, and D.S. Horne, Kinetics of aggregation of as1-casein/Ca 2+ mixtures: charge and temperature effects. *Biophysical Chemistry*, 1981. 13(4): p. 307-314.

8. Parker, T.G. and D.G. Dalgleish, Binding of calcium ions to bovine beta-casein. *Journal of Dairy Research*, 1981. 48: p. 71-76.

9. Thomar, P., et al., Slow dynamics and structure in jammed milk protein suspensions. *Faraday Discussions*, 2012. 158: p. 325-339.

10. Pitkowski, A., T. Nicolai, and D. Durand, Stability of caseinate solutions in the presence of calcium. *Food Hydrocolloids*, 2009. 23(4): p. 1164-1168.

11. Molins, R.A., Phosphates in milk and dairy products chapter 4, in Phopshates in food, C. Press, Editor. 1991.

12. Carr, A.J., C.R. Southward, and L.K. Creamer, Protein hydration and viscosity of dairy fluids., in Advanced Dairy Chemistry, Vol. 1, Proteins. 3rd ed, P.F. Fox and P.L.H. McSweeney, Editors. 2003, Kluwer Academic/Plenum Publishers: New York, USA.

13. Aoki, T., Cross-linkage between casein and colloïdal calcium phosphate in bovine casein micelle, in Interactions of food proteins, N.P.a.R. Barford, Editor. 1991. p. 164-181.

14. Aoki, T., et al., Role of individual milk salt constituents in cross-linking by colloidal calcium phosphate in artificial casein micelles. *Agricultural and Chemical Chemistry*, 1987. 51(3): p. 817-821.

15. Aoki, T., et al., Response surface analyses of the effects of calcium and phosphate on the formation and properties of casein micelles in artificial micelle systems. *Journal of Agricultural and Food Chemistry*, 1996. 44: p. 1230-1234.

16. Horne, D.S., Calcium-induced precipitation of Î± S1-casein: effect of inclusion of citrate or phosphate. *Journal of Dairy Research*, 1982. 49(01): p. 107-118.

17. Guo, M., et al., Casein precipitation equilibria in the presence of calciums ions and phosphates. *Colloids and Surfaces B: Biointerface*s, 2003. 29: p. 297-307.

18. Alvarez, E.M., et al., Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate. *Colloid & Polymer Science*, 2007. 285(5): p. 507-514.

19. Zittle, C.A. and E.S. Dellamonica, Binding of calcium to casein: influence of pH and calcium and phosphate concentrations. *Archives of Biochemistry and Biophysics*, 1958. 76: p. 342-353.

20. Zittle, C.A., E.S. Della Monica, and J.H. Custer, Effect of Certain Salts on Precipitation of Casein by Calcium Chloride and Heat. *Journal of Dairy Science*, 1957. 40(3): p. 280-288.

21. Gonzalez-Jordan, A., et al., The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution. *Food Hydrocolloids*, 2015.

22. Heber, A., et al., 31P NMR spectroscopic investigations of caseins treated with microbial transglutaminase. *Food Hydrocolloids*, 2012. 28(1): p. 36-45.

23. Rulliere, C.l., et al., Studies of polyphosphate composition and their interaction with dairy matrices by ion chromatography and ^{31}P NMR spectroscopy. *International Dairy Journal*, 2013. 28(2): p. 102-108.

24. Miranda, G., et al., Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics*, 2004. 4(8): p. 2496-2509.

25. Thomar, P. and T. Nicolai, Dissociation of native casein micelles induced by sodium caseinate. *Food Hydrocolloids*, 2015. 49: p. 224-231.

26. Pitkowski, A., D. Durand, and T. Nicolai, Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science*, 2008. 326(1): p. 96-102.

27. De Kort, E., et al., Calcium-binding capacity of organic and inorganic ortho-and polyphosphates. *Dairy Science and Technology*, 2009. 89(3-4): p. 283-299.

28. Mekmene, O., et al., Effects of pH and Ca/P molar ratio on the quantity and crystalline structure of calcium phosphates obtained from aqueous solutions. *Dairy Science and Technology*, 2009. 89(3-4): p. 301-316.

29. Silva, N.N., et al., pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties. *Food Hydrocolloids*, 2013. 32(2): p. 322-330.

Chapter 8. The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution.

The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution.

Published in *Food Hydrocolloids* (2015), 51 88-94

Alberto Gonzalez-Jordan, Peggy Thomar, Taco Nicolai, Jens Dittmer

LUNAM, Université du Maine, IMMM UMR CNRS 6283, PCI, 72085 Le Mans Cedex 9, France

Email : Taco.nicolai@univ-lemans.fr

Abstract

The mobility of phosphate groups in aqueous solutions of casein micelles and sodium caseinate from bovine milk was determined with magic angle spinning (MAS) 31P NMR as a function of the pH between pH 4 and pH 8. The chemical shifts and the relative amplitudes of the signals from mobile inorganic phosphate (orthophosphate) and mobile organic phosphate (phosphorylated serines) as well as that of immobile phosphate (colloidal calcium phosphate, and immobile phosphorylated serines) were determined. Sodium caseinate contained very little orthophosphate and all phosphates were mobile over the whole pH range. In micellar casein solutions most of the phosphate was immobile at pH > 6, but the fraction of mobile organic and inorganic phosphate increased sharply between pH 5.5 and pH 4.5, showing the disintegration of the CCP nanoclusters. Protonation of the phosphates with decreasing pH was determined from the chemical shift and was related to their mobility. The signal of mobile organic phosphate was different for micellar casein solutions and sodium caseinate demonstrating the influence of calcium phosphate in the former. The microscopic structure of protein solutions was investigated with Confocal Laser Scanning Microscopy. Large protein clusters were observed below pH 5.2 with a density that increased with decreasing pH down to pH 3.9. The mobility of either organic or inorganic phosphate at pH 6.8 was not significantly different after the pH had been reduced to 4.8 and subsequently increased to 6.8, but the microstructure was strongly influenced by the pH-cycling.

1. Introduction

Casein is the major protein component of cow's milk, representing about 80% of its protein content. Its structure and behaviour in aqueous solution have been investigated intensively for decades, (see Dalgleish, 2011; De Kruif, 2014; De Kruif, Huppertz, Urban and Petukhov, 2012; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2009) for recent reviews, but are still not fully understood. Bovine casein consists of mainly four types: α_{s1}, α_{s2}, β and κ, and in milk the majority of casein is assembled into spherical aggregates, so-called casein micelles, with an average radius of about 100 nm. Currently, the generally accepted view is that the casein micelles contain nanoclusters of calcium orthophosphate, so-called colloidal calcium phosphate (CCP), that are distributed throughout the micelles. Phosphorylated serines (SerP) in the caseins bind to the nanoclusters or even become an integrated part of the CCP, which maintains the integrity of the micelles together with attractive interactions between the casein chains (Holt et al., 2013). The size of the casein micelles is limited by κ-casein that forms a polyelectrolyte brush at the surface of the micelle. Steric hindrance of the κ-casein layer together with electrostatic repulsion inhibits aggregation of the micelles in milk. The net charge of the casein micelles decreases with decreasing pH from the value in milk (pH 6.7) towards its iso-electric point (pI≈4.6). Acidification also leads to progressive protonation of organic and inorganic phosphate and causes progressive dissolutionof the CCP until all phosphate is solubilized at pH < 5.3 (Dalgleish & Law, 1989; Famelart, Lepesant, Gaucheron, Le Graet, & Schuck, 1996; Le Graët & Brulé, 1993; Marchin, Putaux, Pignon, & Leonil, 2007). It was observed that the size of casein micelles in unheated milk decreases only very little with decreasing pH until pH 5.0 (Anema, Lowe, & Lee, 2004; Dalgleish, Alexander, &Corredig, 2004; De Kruif, 1997; Moitzi, Menzel, Schurtenberger, & Stradner, 2010). At lower pH, the proteins aggregate leading to precipitation or gelation, which is the basis for yoghurt formation. Even though the casein micelles remain largely intact, at least down to pH 5.0, their internal structure and the interaction between the caseins change during acidification as the CCP progressively dissolves. It is clear that in order to understand this process fully it is necessary to investigate the state of both the organic and inorganic phosphate as a function of the pH. ^{31}P NMR spectroscopy is a non-destructive technique that can be used to quantify the degree of protonation of the phosphoserine of casein as a function of the pH (Belton, Lyster, & Richards, 1985; Humphrey & Jolley, 1982; Sleigh, Mackinlay, & Pope, 1983). It was shown with this technique that the majority of organic (P_o) and inorganic (P_i) phosphorus in micellar casein solutions is immobile at pH 6.7 and that it can be characterized by magic angle spinning (MAS) NMR (Bak,

Rasmussen, Petersen, & Nielsen, 2001; Rasmussen, Sørensen, Petersen, Nielsen, & Thomsen, 1997; Thomsen, Jakobsen, Nielsen, Petersen, & Rasmussen, 1995). However, as far as we are aware, no systematic investigation has been made so far of the mobility of P_o and P_i in aqueous solutions of casein micelles as a function of the pH. Here we report on a MAS ^{31}P NMR investigation of aqueous solutions of casein micelles in the form of native phosphocaseinate over a wide pH-range (4-8). Our aim was to determine quantitatively the fraction of mobile P_o and P_i as a function of the pH in order to trace the dissolution of the CCP. We also determined the degree of protonation of the mobile phophoserine and inorganic phosphate and compared it with that of caseins without CCP, i.e. sodium caseinate (NaCas). The effect of decreasing the pH on the microscopic structure of the casein solutions was visualized by Confocal Laser Scanning Microscopy (CLSM). Finally, we investigated the effect of pH-cycling on the mobility of P_i and P_o and the microscopic structure for a micellar casein solution at pH 6.8 before and after acidification. In this investigation we have studied the effect of acidification on the state of organic and inorganic phosphate for casein in pure water. The influence of the presence of minerals on the effect of acidification will be addressed in a future investigation.

2. Materials and methods

Commercial NaCas powder (Lactonat EN) was provided by Lactoprot Deutschland GmbH (Kaltenkirchen, Germany). It contained 90% (w/w) protein (TNC, Kjeldahl) and 1.3% (w/w) sodium and 0.7wt% phophorus. Pure α_s and β-casein powders were purchased from Sigma6Aldrich (St. Louis, USA). Micellar casein in the form of native phosphocaseinate powder (NPCP) was obtained by micro- and diafiltration and was provided by INRA-STLO, (Rennes, France). The powder contained 83% (w/w) of protein (TNC, Kjeldahl), 2.6% (w/w) calcium and 1.7% (w/w) phosphorus. The casein composition of the samples was obtained using reverse phase high pressure liquid chromatography. The fractions of α_{s1}-, α_{s2}-, β-, and κ-casein in NaCas were 34, 3, 44, 9%, respectively. In addition, the sample contained 9% unidentified protein, most likely partially degraded casein. The casein composition of caseins in NPCP was approximately the same. Considering that the amount of P_i in NaCas is very small it follows from the phosphorus content that the fraction of P_i and P_o in the casein micelle powder used for this study was 0.6 and 0.4 respectively, in agreement with that reported by Famelart et al. (1996).

2.1. Sample preparation. NaCas and NPCP were dissolved while stirring in deionised water (Millipore) containing 3 mM sodium azide as a bacteriostatic agent. NaCas solutions were heated at 80°C for 30 min and NPCP solutions were heated at 50°C for 16 h in order to obtain fully hydrated homogeneous suspensions. The protein concentration was determined by absorption of UV-light withwavelength 280 nm (Varian Cary-50 Bio, Les Ulis, France) assuming an extinction coefficient of 0.81 L.g. cm^{-1}. The pH was adjusted by dropwise additionof concentrated 0.1-1M NaOH or HCl solutions while vigorously stirring. All experiments shown here were done at a fixed casein concentration after pH adjustment of C=100g.L^{-1}, except the pure α-casein solution for which C =75 g.L^{-1}. The fraction of proteins in the form of micelles was determined by centrifugation at $5.10^4 g$ during 2 h at 20 °C using an ultracentrifuge (Beckman Coulter, Allegra 64R, Villepinte, France). About 15% of the proteins did not sediment in these conditions. Further dissolution of casein micelles was extremely slow at this high protein concentration and was negligible over the duration of the experiments.

2.2. Nuclear magnetic resonance experiments. The NMR experiments were conducted on a Bruker Avance III 300 MHz WB spectrometer equipped with a 4 mm MAS VTN type probe head with two channels. An HR/MAS rotor was filled with about 50 µL of a casein or caseinate solution at C =100 g.L^{-1} and spun at 3 kHz. ^{31}P direct excitation spectra were accumulated over between 128 and 1024 repetitions with a relaxation delay of 30 s, an acquisition time of 0.2 s, and a ^{1}H decoupling with reduced power (8.5 kHz) in order to avoid probe head damage during the relatively long acquisition needed for adequate sampling of relatively narrow signals. In addition, ^{1}H-^{31}P cross-polarization (CP) spectra have been acquired with 6144 repetitions with a relaxation delay of 5 s in order to characterize the broad signal from immobile phosphorus. All spectra have been normalized with respect to the H_2O ^{1}H signal in order to account for the variation in the sample volume in the HR/MAS rotor. The line width and position of the broad signal from immobile P were determined from the complementary crosspolarization spectra. The mobile signals from inorganic and organic phosphate were quantified by deconvolution and integration of the corresponding peaks in the ^{31}P direct excitation spectra after subtraction of the immobile P signal. The spectra have been referenced to an aqueous solution of H_3PO_4.

2.3. Confocal laser scanning microscopy. Confocal laser scanning microscopy (CLSM) images were made with a Leica TCS-SP2 (Leica Microsystems, Heidelberg, Germany) using a water immersion objective lens HCx PL APO 63x NA = 1.2. The caseins were labelled with the fluorochrome rhodamine b by adding a small amount of a concentrated rhodamine solution for a final concentration of 5ppm in the solutions. The rhodamine was excited using a

heliumeneon laser with wavelength 543nm and the fluorescence was detected with a photomultiplier. Care was taken not to saturate the fluorescence signal and it was verified that the amplitude of the signal was proportional to the protein concentration.

3. Results and discussion

We will first discuss the effect of acidification of aqueous solutions of NaCas at C=100 $g.L^{-1}$ that contains only trace amounts of calcium and inorganic phosphate. Then we will show the results for acidification of micellar casein solutions at the same protein concentration and compare them with those obtained for NaCas.

3.1. Sodium caseinate

Figure 1 shows the direct excitation ^{31}P MAS NMR spectrum of NaCas at pH 6.8. It contains a relatively small narrow peak at δ =1.2ppm and a broader signal with larger amplitude at δ =2.5ppm. The narrow peak was due to free orthophosphate that was present in a small amount in the NaCas sample. The broad signal was due to organic phosphate from the phosphoserine residues (SerP). One can distinguish three peaks in the P_o signal as was earlier reported by (Kakalis, Kumosinski, & Farrell, 1990) for NaCas at pH 7.6 and by (Heber, Paasch, Partschefeld, Henle, & Brunner, 2012) for milk after addition of EDTA. For comparison we also show in Figure 1 spectra of purified α_s- and β-casein solutions at pH 6.8. The signal of NaCas is very close to the average of α_s- and β-casein signals weighted in proportion to their content in the NaCas sample, see dashed line in Figure 1. The contribution of κ-casein to the NMR signal is negligible (≈2%), because it contains only one SerP. α_{s1}-, α_{s2}-and β-casein contain 8-9, 11-13 and 5 SerP per chain, respectively, and it is clear that the peaks observed in the spectra are not to distinct signals from each phosphoserine. However, attempts have been made to assign the peaks to different groups of SerP with similar environment within the casein chain (Humphrey et al., 1982; Sleigh et al., 1983). We note that the narrow signal at 1.3ppm was absent for the purified casein samples, but appeared when we added 1mM Na_2HPO_4, which shows without ambiguity that this peak is caused by inorganic phosphate.

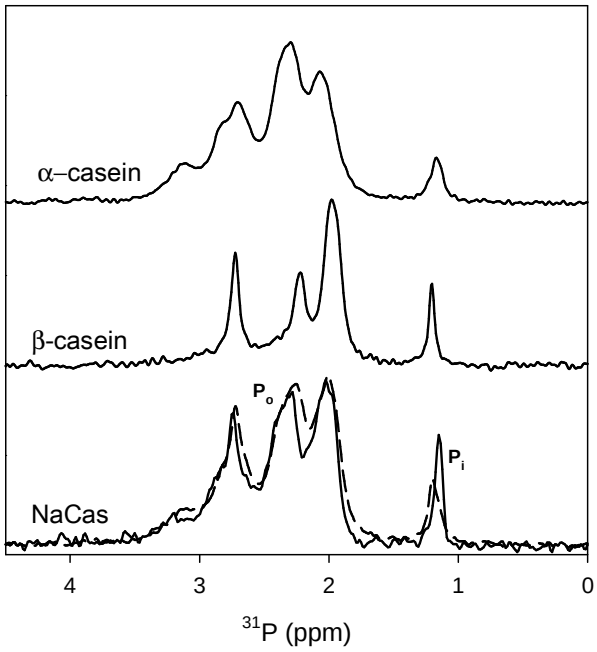

Figure 1. ^{31}P MAS NMR spectra of aqueous solutions of NaCas and purified α_s- and β-casein at pH 6.8. 1mM Na_2HPO_4 was added to the purified casein solutions. The dashed line in the spectrum of NaCas corresponds to the weighted average of the spectra of purified α_s- and β-casein.

^{31}P MAS NMR spectra of NaCas at different pH are shown in Figure 2. An upfield shift of both the P_o and P_i signals was observed when the pH decreased. The P_i signal remained narrow over the whole pH-range, but the width of the broad P_o signal group taken at half its maximum amplitude decreased with increasing pH above pH 7.2 and with decreasing pH below pH 6.8. The three peaks of the P_o signal can be clearly distinguished only in a narrow range of the pH between 6.6 and 8.0. The dependence of δ on the pH of the narrow P_i signal and the average P_o signal is shown in Figure 3. The width of the P_o signal is indicated by bars.

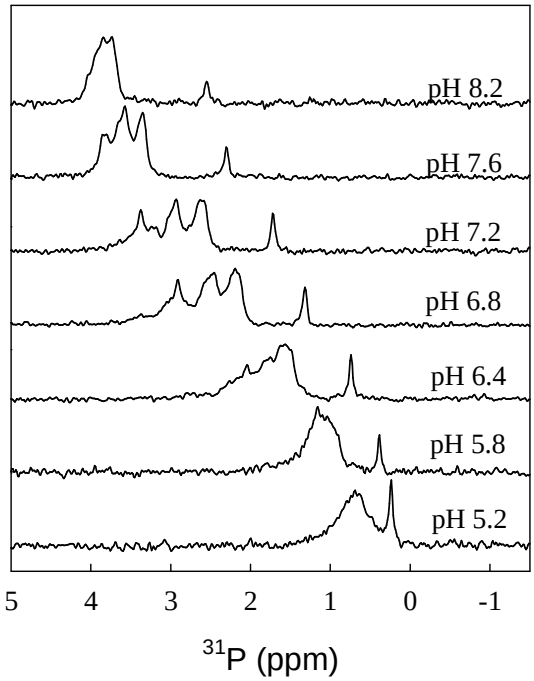

Figure 2. ^{31}P MAS NMR spectra of aqueous solutions of NaCas at different pH indicated in the figure.

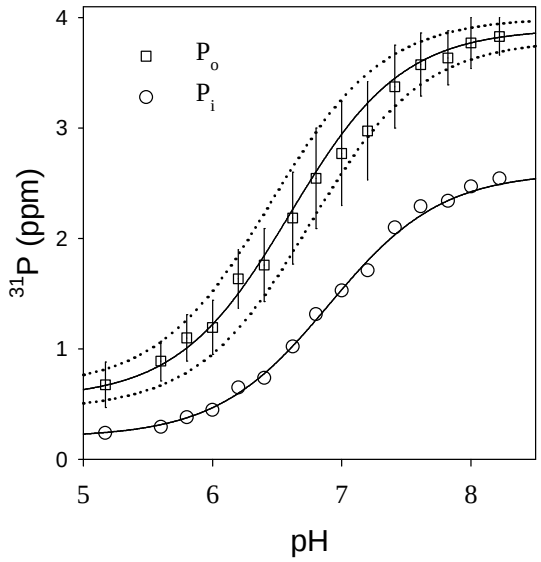

Figure 3. pH-dependence of the chemical shift of organic and inorganic phosphorus. The bars indicate the width of the P_o signal. The solid and dotted lines represent fits with Eq.(1).

The upfield shift of δ with decreasing pH is caused by increased shielding of the P nuclei as consequence of protonation of the phosphates. The pH dependence of δ can thus be used to calculate the equilibrium constant of the second protonation of the phosphates.

The chemical shift as a function of the pH was analysed assuming that protonation of each phosphate is characterized by a single pKa. In that case the relationship between the pH and the chemical shift is as follows:

$$\delta = (\delta_1 X + \delta_2)/(X+1) \quad (1)$$

where δ_1 and δ_2 are the chemical shifts of protonated and deprotonated phosphate, respectively, and $X = 10^{pH-pKa}$ is the molar ratio between protonated and deprotonated phosphate. Only an average signal is observed because the exchange of protons between different phosphates is fast compared to the NMR time scale.

The narrow signal of P_i and the average P_o can be well described by Eq. (1), see solid lines in Figure 3. For P_i we find pKa =7.0 (δ_1=0.2 and δ_2=2.6 ppm) which is slightly smaller than the literature value for the second protonation of pure orthophosphate (pKa = 7.2). For the average chemical shift of the organic phosphate we find pKa = 6.6 (δ_1=0.5 and δ_2=3.9). However, phosphoserines with different environments have slightly different pKa values, which caused the dispersion of the P_o signal. The variation of the width of the signal from the organic phosphate as a function of the pH can be explained by a pKa range from about 6.45 to 6.8, see dotted lines in Figure 3. The pKa values obtained for P_o in this study are within the range (5.8-6.9) that has been reported for purified caseins in the literature (Belton et al., 1985; Humphrey et al., 1982).

3.2. Casein micelles

The direct excitation ^{31}P MAS NMR spectrum of a micellar casein solution at pH 6.8 shows one narrow peak at δ= 1.5 ppm, a broader peak at 3.2ppm and a very broad peak centred at 2.5ppm, see Figure 4. Similar spectra have been reported for micellar casein solutions in the literature (Andreotti, Trivellone, & Motta, 2006; Bak et al., 2001; Belton et al., 1985; Heber et al., 2012; Rasmussen et al., 1997; Thomsen et al., 1995). The narrow peak at 1.5ppm is due to free orthophosphate in solution, while the peak at 3.2ppm can be assigned to mobile SerP. The latter is broadened by the different chemical environments of the SerP as was discussed above for NaCas. However, the shape of this peak is different from that of NaCas which means that the environment of the mobile SerP was different. The very broad peak at 2.5ppm originated from immobile phosphate (Bak et al., 2001; Rasmussen et al., 1997; Thomsen et al., 1995) both organic and inorganic. This can be confirmed by cross polarisation NMR that probes specifically the signal from immobile phosphate, see dashed line in Figure 4. A few authors

have analysed this signal in more detail and distinguished different types of immobile phosphate (Bak et al., 2001; Rasmussen et al., 1997; Thomsen et al., 1995), but we will not attempt this here. The relative amplitude of the signals was obtained by deconvolution of the spectrum, yielding 2%, 7.5% and 90.5% for mobile P_i, mobile P_o, and immobile P, respectively. The fraction of P_i and P_o in the casein micelle powder used for this study was 0.4 and 0.6, respectively, see Materials and Methods. This means that 97% of P_i and 81% of P_o is immobile. Most likely both the immobile organic and the inorganic phosphate form the CCP nanoclusters. We note that no significant difference was observed between spectra obtained for freshly prepared samples and after standing for 1 month at 4°C implying that the casein micelles were stable at the protein concentration used in the study.

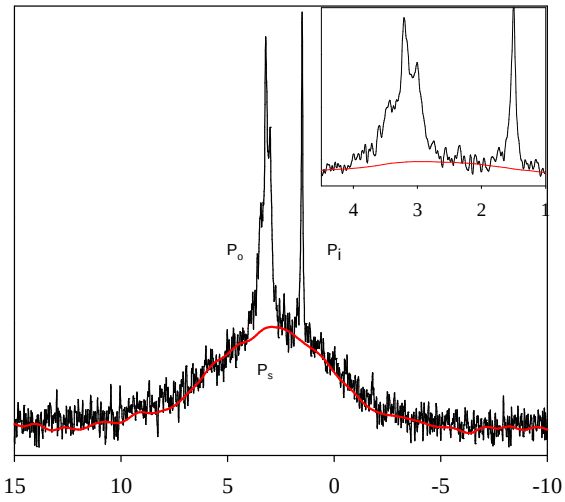

Figure 4. ^{31}P MAS NMR spectrum of an aqueous solution of casein micelles at pH 6.8. The red dashed line represents the cross-polarization spectrum. The insert shows a closeup of the mobile P_o and P_i signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ultracentrifugation (5.10^4 *g*, 1 h) leads to sedimentation of casein micelles, while NaCas remains in the supernatant. When an aqueous NPCP solution at pH 6.8 was centrifugated only 85-90% of the casein sedimented, which means that 10-15% of the casein was not incorporated into the micelles. This may in part explain why 20% of P_o was mobile. However, the fraction of casein in the supernatant was found to decrease with decreasing pH in accordance with results obtained by Famelart et al. (Famelart et al., 1996) for a different batch of NPCP, while the fraction of mobile P_o increased as we will show below. Therefore one cannot relate the fraction of mobile P_o directly to the fraction of non-sedimentable casein.

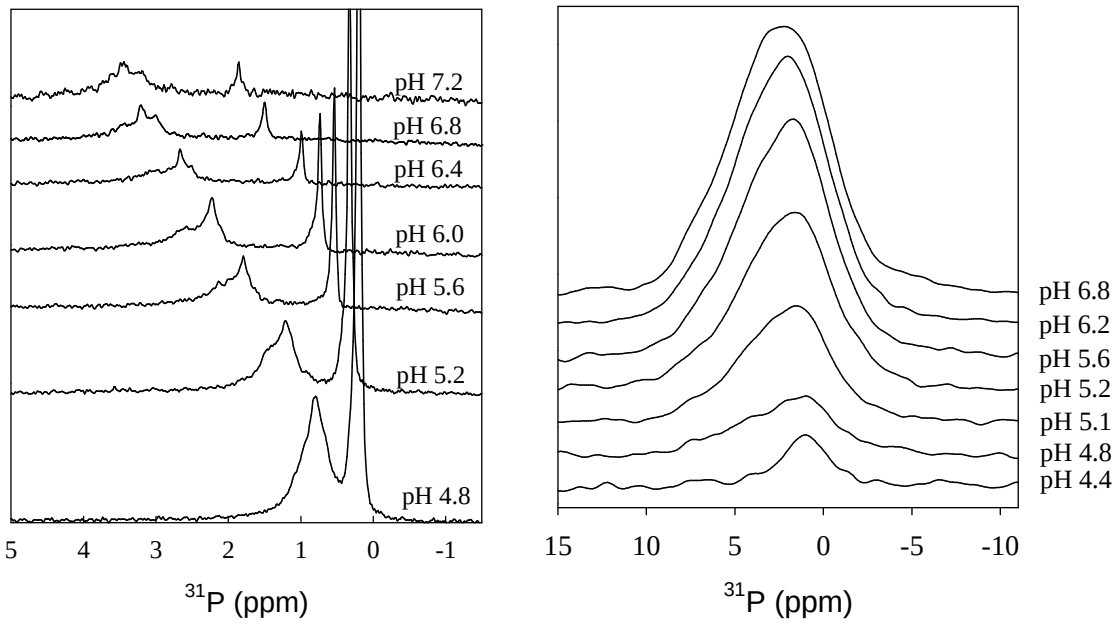

Figure 5. ^{31}P MAS NMR (left panel) and cross-polarization NMR (right panel) spectra of aqueous solutions of casein micelles at different pH. For clarity the spectra at different pH have been shifted vertically.

Figure 5 shows the ^{31}P MAS NMR and cross-polarization NMR spectra of aqueous micellar casein solutions at different pH. As was observed for NaCas, the chemical shift of the mobile P_i and P_o signals moved upfield when the pH was decreased. In addition, the amplitude of these signals increased strongly below pH 6. The chemical shift of mobile P_o and P_i in micellar casein solutions as a function of the pH is compared to that in NaCas solutions in Figure 6. It is clear that compared to NaCas the sharp upfield shift with decreasing pH occurs at lower pH for the casein micelles especially for P_o. Models assuming a single equilibrium constant yield pKa=5.8 (δ_1=0.5, δ_2=3.6 ppm) for P_o and pKa=6.5 (δ_1=0.2, δ_2=2.2 ppm) for P_i. However, the assumption of a single pKa describes the data less well for the casein micelles than for NaCas. The shift of the protonation of the mobile phosphate groups to lower pH entails that their environment was different as is also evident from the different shape of the NMR signal. In addition, the environment changes with decreasing pH, which may explain why the model assuming a single pKa was not as good as for NaCas. The most likely reason for the different pH-dependence of the chemical shift is that the micellar casein solutions contain calcium and orthophosphate in the ionic form in increasing quantities as the pH is decreased as a consequence of the CCP being dissolved. These ions can screen electrostatic interactions and interact specifically with SerP. The relative amplitudes of the different phosphorus signals were determined by deconvolution

of the single pulse spectra and from the peak integral in the CP spectra shown in Figure 5. Figure 7a shows that the fraction of immobile phosphate decreased sharply for pH < 6 and became very small for pH < 4.5. The fraction of mobile organic and inorganic phosphate is plotted as a function of the pH down to pH 4.8 in the inset of Figure 7a. At lower pH, the P_i and P_o signals overlapped strongly and could not be reliably separated. In Figure 7b we have plotted the variation of the fraction of mobile P_i and P_o with respect to the total amount of each. At pH > 6 almost 20% of the organic phosphate was mobile whereas less than 10% of the inorganic phosphate was mobile. The fraction of mobile phosphate increased rapidly below pH 5.5 and reached 75% at pH 4.8 for both P_i and P_o.

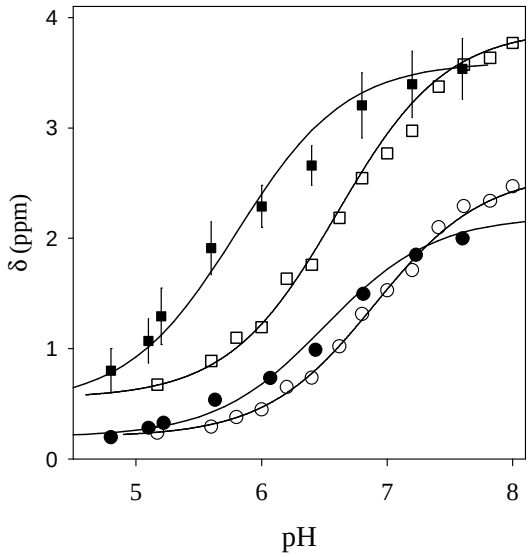

Figure 6. pH-dependence of the chemical shift of organic (squares) and inorganic (circles) phosphorus in aqueous solutions of casein micelles (filled symbols) and NaCas (open symbols). The bars indicate the width of the P_o signal for the casein micelles. The solid lines represent fits corresponding to Eq. (1).

Le Graët & Gaucheron (1999) determined the fraction of 'soluble' inorganic phosphate in solutions of NPCP in milk ultrafiltrate as a function of the pH. Soluble phosphate was defined as the phosphate that passed a filter with a 25 kDa cut-off membrane. They showed that the fraction of soluble phosphate increased rapidly for pH < 5.5 and depended weakly on the casein concentration. The fraction of solubilized inorganic phosphate obtained in this manner at the casein concentration used here is compared with the fraction of mobile P_i, see solid line Figure 7b. Qualitatively the pH-dependence is the same, but the sharp increase of the fraction of soluble P_i reported by Le Graët & Gaucheron (1999) started at slightly lower pH than the increase of mobile P_i observed here. This may be due to the fact that they prepared the casein micelles

solutions in milk ultrafiltrate while we have prepared them in pure water. Alternatively, it may mean that a fraction of the soluble P_i was immobile at these pH values.

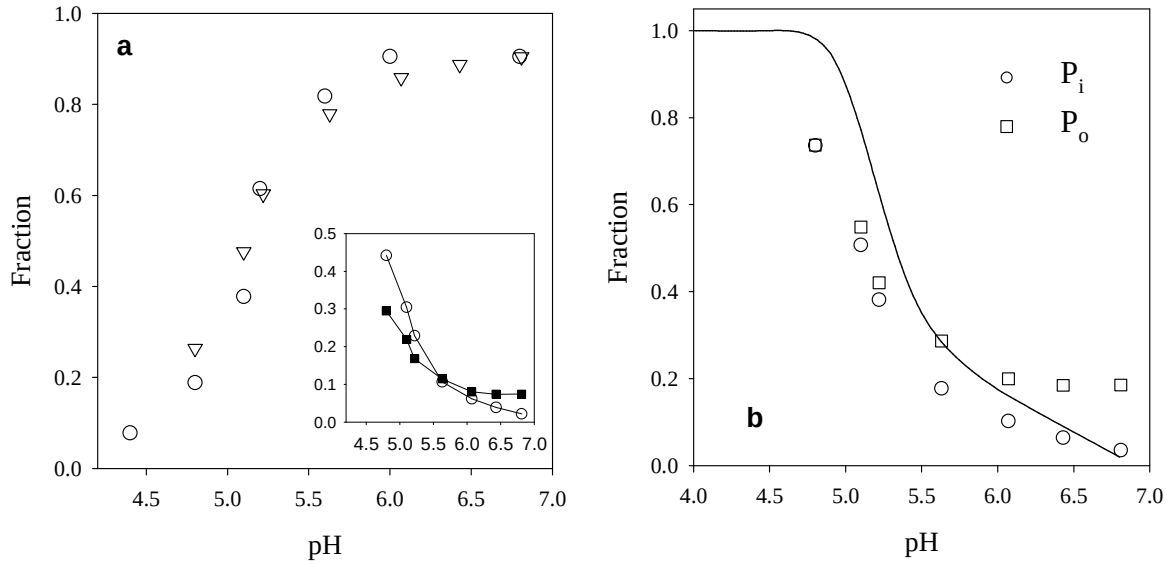

Figure 7. (a) Fraction of immobile phosphate as a function of the pH in aqueous solutions of casein micelles obtained from single pulse (triangles) and cross-polarization experiments (circles). The in-set shows the fraction of mobile organic (filled symbols) and inorganic (open symbols) phosphate. (b) Fraction of mobile organic (squares) and inorganic (circles) phosphate with respect to the total organic and inorganic phosphate, respectively. The solid line represents the fraction of soluble inorganic phosphate taken from ref. Le Graët & Gaucheron (1999).

Visual inspection of the samples showed that a significant fraction of the proteins in casein micelles solutions below pH 5.0 formed large flocs that sedimented slowly under gravity. This observation is consistent with results by Famelart et al. (1996) who reported for NPCP solutions in pure water a sharp increase of the fraction of casein that sedimented after centrifugation for 1 h at $10^3 g$ from less than 5% for pH ≥ 5.2 to about 90% for pH≤5.0. The CLSM images in Figure 8 show the formation of microscopic casein flocs after acidification starting at pH 5.2 with a density that increased sharply with decreasing pH. Remarkably, large scale aggregation of the caseins did not render the SerP groups immobile, but, on the contrary, the fraction of mobile phosphate continued to increase progressively below pH 5.2. The large scale flocculation of the caseins does not seem to be directly related to the state of the phosphate.We notice that NaCas also precipitates or gels below pH 5.2 (Braga, Menossi, & Cunha, 2006; HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Lucey, Van Vliet, Grolle, Geurts, & Walstra, 1997; O'Kennedy, Mounsey, Murphy, Duggan, & Kelly, 2006; Ruis, Venema, & van der Linden, 2007).

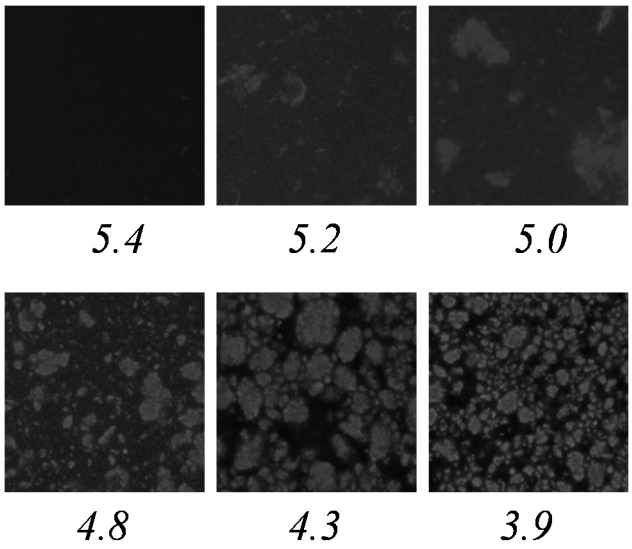

Figure 8. CLSM images (150×150 mm) of micellar casein solutions at different pH indicated in the figure.

It is clear that dissolution of the CCP by lowering the pH releases inorganic phosphate and calcium from the CCP and renders SerP mobile. Silva et al. (2013) dialysed micellar casein solutions at different pH between 6.7 and 5.5 against milk ultrafiltrate in order to remove the released calcium phosphate. They subsequently increased the pH back to 6.8 and determined the fraction of nonsedimentable casein after ultracentrifugation. The fraction of nonsedimentable casein was found to increase from 25% at pH 6.7 to 70% at pH 5.5. It was also found that the fraction of inorganic phosphate that was removed by dialysis increased with decreasing pH up to 60% at pH 5.5. The fraction of dissociated casein and solubilized P_i at pH 5.5 was significantly larger than the fraction of mobile P_o and P_i at this pH found in the present study, which may be due to the extended dialysis. Nevertheless, these experiments show that demineralization of casein micelles by acidification leads to dissociation of a fraction of the casein micelles at neutral pH where the charge density of the caseins is higher. However, as we mentioned above, if the system is maintained at lower pH the caseins remain aggregated and sediment during ultracentrifugation. This means that the reduced stabilizing effect of CCP nanoclusters is compensated at lower pH by other types of attractive interactions between the casein chains (Holt et al., 2013). When the charge density of the caseins is increased by increasing the pH these interactions are not sufficient to maintain the integrity of the micelles in the absence of a large fraction of the calcium phosphate.

3.3. pH cycling

An interesting question is whether the mobilization of phosphates by acidification is reversible upon increasing the pH. Figure 9 shows the ^{31}P MAS NMR spectrum of a micellar casein solution of which the pH was first reduced from 6.8 to 4.8, which rendered most of the phosphates mobile (compare Figure 5), and was subsequently increased back to 6.8. Comparison of the spectra of the solution at pH 6.8 before and after pH-cycling, shows that the signals were almost the same except that the amplitude of the mobile P_i signal was slightly larger before cycling. The signal of residual mobile P_o was similar before and after pH-cycling, but not identical. Clearly most of the phosphate was immobilized when the pH was raised, but no calcium phosphate particles were visible on length scales accessible to CLSM. Most likely growth of the calcium phosphate clusters was limited by incorporation of SerP, which rendered the latter immobile. Holt,Wahlgren, & Drakenberg (1996) observed the formation of amorphous dicalcium phosphate nanoclusters from an undersaturated solution of salts and the phosphopeptide (β 1-25) by raising the pH slowly from 5.5 to 6.7. The ^{31}P NMR spectrum at pH 6.7 in that study contained a very broad signal that represented 75% of the total signal implying that 75% of the phosphate had been immobilized by the formation of the nanoclusters. In addition to the broad signal they observed a narrow signal from mobile inorganic phosphate and a broader three peaked signal from mobile organic phosphate.

It appears that also for micellar casein solutions studied here, nanoclusters were formed when the pH was increased, but we have no information on the size of the clusters nor on their structure. Therefore we do not know if they are similar to the ones in native casein micelles. In any case, CLSM images of the solution before and after pH-cycling showed that the structure on larger length scales was different, see Figure 9. Before pH-cycling the system was homogeneous on microscopic length scales, while after pH-cycling we observed large flocs, albeit with a much lower density than at pH 4.8. The acid-induced aggregation of micellar caseins is thus not reversible upon increasing the pH contrary to the acid-induced aggregation of NaCas.We note that the presence of large flocs after pH-cycling causes some uncertainty in the amount of casein of this sample in the NMR probe and thus the total amplitude of the signal. We may speculate that the internal structure of the large flocs is similar to that of the casein micelles. Calcium phosphate nanoclusters incorporating phosphoserines of α- and β-caseins are bound to other caseins via attractive interactions between the proteins. The main difference with the structure of native casein micelles is the role of κ-casein that did not form a stabilizing layer

that inhibits growth of the casein/nanocluster aggregates during formation of the native micelles. This may be due to the fact that casein was already aggregated at pH 4.8 and that the protein concentration used in this study was relatively high. Further investigation employing scattering techniques and electron microscopy are needed to establish to what extent the local structure of the casein assemblies after pH-cycling resembles that of native casein micelles.

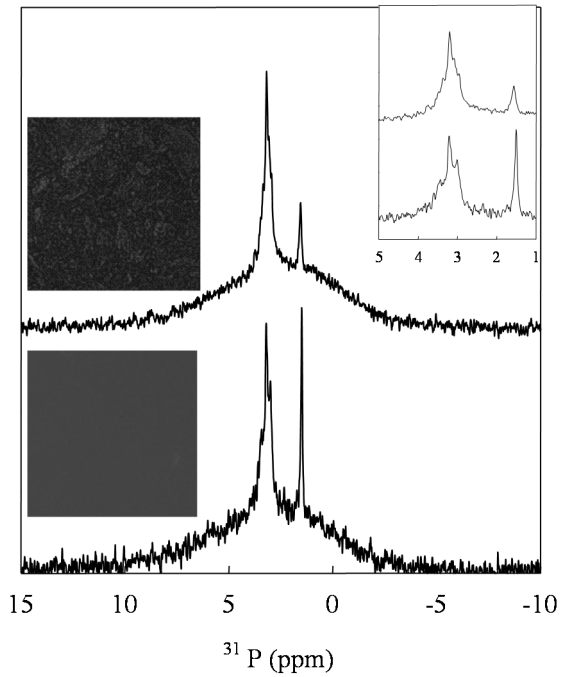

Figure 9. ^{31}P MAS NMR spectra of aqueous solutions of casein micelles at pH 6.8 before (bottom) and after (top) pH-cycling to pH 4.8. The insert shows a close-up of the signals of mobile P_o and P_i. CLSM images of the corresponding solutions are also shown.

4. Conclusions

Magic angle spinning ^{31}P NMR allows one to determine quantitatively the fraction and the protonation of mobile organic and inorganic phosphate in casein solutions as a function of the pH in the range 4.8-8.0. The organic phosphate of sodium caseinate in aqueous solution is mobile over the whole pH range even for pH < 5.2 where the caseinate precipitates during centrifugation and microscopic flocs are observed in CLSM image. The ^{31}P NMR signal of sodium caseinate at pH 6.8 is equal to the weighted average of purified α- and β-caseins implying that the local environment of the phosphoserines is not very different from that in the pure α and β-casein aggregates. The average pKa value of NaCas was 6.6, but varied slightly for different SerP on the caseins, which caused the appearance of distinct peaks around pH 7.

For casein micelles in aqueous solution at the pH of milk, 97% of the inorganic phosphate and 81% of the organic phosphate is immobile and most likely incorporated in calcium phosphate nanoclusters. The fraction of mobile organic and inorganic phosphate increased weakly with decreasing pH down to pH 5.5 and then increased sharply to reach 75% at pH 4.8. Protonation of the inorganic and organic phosphates was shifted to lower pH and for both it was less well described by Eq. (1) than for NaCas solutions, demonstrating the influence of the minerals present in micellar casein solutions. In spite of the release of the phosphates from the nanoclusters by lowering the pH, most of the proteins sedimented during ultracentrifugation at all pH and large flocs were observed for pH < 5.2. There is no correlation between the mobility of the phosphates and the solubility of the caseins.

Calcium phosphate nanoclusters that were dissolved by lowering the pH to 4.8 reformed when the pH was increased again to 6.8 and the fraction of immobile phosphate was almost the same before and after pH-cycling. However, the microscopic structure of the micellar casein solutions was different as after pH-cycling microscopic casein clusters were visible with CLSM. Possibly the local organisation of the caseins and the calcium phosphate is similar before and after pH-cycling, but the stabilizing role of κ-casein that limits growth during formation of the native micelles is absent after pH-cycling.

References

Andreotti, G., Trivellone, E. & Motta, A. (2006), Characterization of buffalo milk by ^{31}P-nuclear magnetic resonance spectroscopy, *Journal of Food Composition and Analysis*, 19, 843-849.

Anema, S. G., Lowe, E. K. & Lee, S. K. (2004), Effect of pH at heating on the acid-induced aggregation of casein micelles in reconstituted skim milk, *LWT-Food Science and Technology*, 37, 779-787.

Bak, M., Rasmussen, L. K., Petersen, T. E. & Nielsen, N. C. (2001), Colloidal calcium phosphates in casein micelles studied by slow-speed-spinning 31P magic angle spinning solid-state nuclear magnetic resonance., *Journal of Dairy Science*, 84, 1310-1309.

Belton, P. S., Lyster, R. L. & Richards, C. P. (1985), The ^{31}P nuclear magnetic resonance spectrum of cows' milk, *Journal of Dairy Research*, 52, 47-54.

Braga, A., Menossi, M. & Cunha, R. (2006), The effect of the glucono-δ-lactone/caseinate ratio on sodium caseinate gelation, *International Dairy Journal*, 16, 389-398.

Dalgleish, D., Alexander, M. & Corredig, M. (2004), Studies of the acid gelation of milk using ultrasonic spectroscopy and diffusing wave spectroscopy, *Food Hydrocolloids*, 18, 747-755.

Dalgleish, D. G. (2011), On the structural models of bovine casein micelles—review and possible improvements, *Soft Matter*, 7, 2265-2272.

Dalgleish, D. G. & Law, A. J. R. (1989), pH-induced dissociation of bovine casein micelles. II Mineral solubilization and its relation to casein release., *Journal of Dairy Research*, 56, 727-735.

De Kruif, C. (1997), Skim milk acidification, *Journal of Colloid and Interface Science*, 185, 19-25.

De Kruif, C. (2014), The structure of casein micelles: a review of small-angle scattering data, *Applied Crystallography*, 47,

De Kruif, C. G., Huppertz, T., Urban, V. S. & Petukhov, A. V. (2012), Casein micelles and their internal structure, *Advances in Colloid and Interface Science*, 171, 36-52.

Famelart, M. H., Lepesant, F., Gaucheron, F., Le Graet, Y. & Schuck, P. (1996), pH-Induced physicochemical modifications of native phosphocaseinate suspensions: Influence of aqueous phase, *Le Lait*, 76, 445-460.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L. & Moulai-Mostefa, N. (2008), Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering, *Food Hydrocolloids*, 22, 1460-1466.

Heber, A., Paasch, S., Partschefeld, C., Henle, T. & Brunner, E. (2012), 31P NMR spectroscopic investigations of caseins treated with microbial transglutaminase, *Food Hydrocolloids*, 28, 36-45.

Holt, C., Carver, J. A., Ecroyd, H. & Thorn, D. C. (2013), Caseins and the casein micelle: Their biological functions, structures, and behavior in foods, *Journal of Dairy Science*, 96, 6127-6146.

Holt, C., Wahlgren, N. M. & Drakenberg, T. (1996), Ability of a β-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters., *The Biochemical Journal*, 314, 1035-1039.

Horne, D. S. (2009), Casein micelle structure and stability, *Milk proteins: From expression to food*, 133-162.

Humphrey, R. S. & Jolley, K. W. (1982), ^{31}P-NMR studies of bovine beta-casein., *Biochimica et Biophysica Acta*, 708, 294-299.

Kakalis, L. T., Kumosinski, T. F. & Farrell, H. M. (1990), A multinuclear , high-resolution NMR study of bovine casein micelles and submicelles, *Biophysical Chemistry*, 38, 87-98.

Le Graet, Y. & Brulé, G. (1993), Les équilibres minéraux du lait: influence du pH et de la force ionique, *Le Lait*, 73, 51-60.

Le Graët, Y. & Gaucheron, F. (1999), pH-induced solubilization of minerals from casein micelles: influence of casein concentration and ionic strength, *Journal of Dairy Research*, 66, 215-224.

Lucey, J., Van Vliet, T., Grolle, K., Geurts, T. & Walstra, P. (1997), Properties of acid casein gels made by acidification with glucono-δ-lactone. 1. Rheological properties, *International Dairy Journal*, 7, 381-388.

Marchin, S., Putaux, J.-L., Pignon, F. & Léonil, J. (2007), Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle x-ray scattering/ultrasmall-angle x-ray scattering, *The Journal of Chemical Physics*, 126, 045101.

Moitzi, C., Menzel, A., Schurtenberger, P. & Stradner, A. (2010), The pH Induced Sol− Gel Transition in Skim Milk Revisited. A Detailed Study Using Time-Resolved Light and X-ray Scattering Experiments, *Langmuir*, 27, 2195-2203.

O'Kennedy, B. T., Mounsey, J. S., Murphy, F., Duggan, E. & Kelly, P. M. (2006), Factors affecting the acid gelation of sodium caseinate, *International Dairy Journal*, 16, 1132-1141.

Rasmussen, L. K., Sørensen, E. S., Petersen, T. E., Nielsen, N. C. & Thomsen, J. K. (1997), Characterization of phosphate sites in native ovine, caprine, and bovine casein micelles and their caseinomacropeptides: a solid-state phosphorus-31 nuclear magnetic resonance and sequence and mass spectrometric study., *Journal of Dairy Science*, 80, 607-614.

Ruis, H. G., Venema, P. & van der Linden, E. (2007), Relation between pH-induced stickiness and gelation behaviour of sodium caseinate aggregates as determined by light scattering and rheology, *Food Hydrocolloids*, 21, 545-554.

Silva, N. N., Piot, M., de Carvalho, A. F., Violleau, F., Fameau, A.-L. & Gaucheron, F. (2013), pH-induced demineralization of casein micelles modifies their physico-chemical and foaming properties, *Food Hydrocolloids*, 32, 322-330.

Sleigh, R. W., Mackinlay, A. G. & Pope, J. M. (1983), NMR studies of the phosphoserine regions of bovine αs1- and β-casein, *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 742, 175-183.

Thomsen, J. K., Jakobsen, H. J., Nielsen, N. C., Petersen, T. E. & Rasmussen, L. K. (1995), Solid-state magic-angle spinning ^{31}P-NMR studies of native casein micelles., *European Journal of Biochemistry* / FEBS, 230, 454-459.

Chapter 9. Heat-induced gelation of casein micelle and sodium caseinate in aqueous suspensions at different pH

Heat-induced gelation of casein micelle in aqueous suspensions at different pH.

Submitted to Colloid and Surfaces A

Peggy Thomar, Taco Nicolai.

LUNAM, Université du Maine, IMMM UMR-CNRS 6283, Polymères, Colloïdes et Interfaces, 72085 le Mans Cedex 9, France.

Email : Taco.Nicolai@univ-lemans.fr

Abstract.

Heat-induced gelation of casein micelles in aqueous solution was investigated between pH 5.2 and pH 6.7 over a wide range of protein concentrations (C=25-160 $g.L^{-1}$). For C≥40 $g.L^{-1}$ the casein micelles rapidly formed a self-supporting gel above a critical temperature (T_c). At C=160 $g.L^{-1}$, T_c decreased from 90°C at pH 6.5 to 30°C at pH 5.4 and increased with decreasing protein concentration. Oscillatory shear measurements during heating showed that the elastic modulus (G_{el}) of the gels increased strongly with increasing protein concentration, but was insensitive to the pH and the heating temperature except close to T_c where G_{el} decreased sharply with decreasing temperature. The microstructure of the gels was observed by confocal scanning laser microscopy. Heat-induced gelation of casein micelles was compared with that of sodium caseinate solutions free of calcium phosphate.

1. Introduction

In milk, the main protein fraction (80% of total protein) is composed of four different types of casein (α_{s1}, α_{s2}, β and κ-casein) associated into a complex with colloidal calcium phosphate (CCP) called casein micelles [1-5]. Steric and electrostatic repulsion of a κ-casein brush at the surface of the micelles inhibits aggregation of casein micelles in aqueous solution [6-7]. CCP is present in the form of nanoclusters that also incorporates phosphoserine groups of the caseins. Reduction of the pH causes progressive dissolution of the CCP, which is complete at pH 5.2 [8-10]. Recently, we have shown using ^{31}P MAS NMR that solubilization of the CCP by decreasing the pH allows the phosphoserines in the casein micelles to become mobile [11].

In milk, the presence of CCP is necessary to maintain the integrity of the micelles, but they remain intact with approximately the same size down to pH~5.1 [12-16]. At low pH the net charge density of the caseins and thus the electrostatic repulsion is small so that hydrophobic and other physical interactions are sufficient to maintain the integrity of the micelles. However, below pH 5.1, electrostatic and steric repulsion between the micelles is no longer sufficient to inhibit aggregation leading to precipitation or gelation [9, 11, 17-18].

In milk, the micelles aggregate when they are heated above 100°C [19], but at lower temperatures they are stable. Nevertheless, preheating of milk above 70°C has a major effect on the acid-induced gelation of casein micelles as it leads to gelation at higher pH and to stiffer gels and has been investigated extensively {Lucey, 1998 #693}. The effect is principally attributed to heat-induced denaturation and aggregation of the globular whey proteins that are present in the serum of milk. It was found that denatured whey proteins co-aggregate with κ-casein into complexes that are in part bound to the casein micelles [20]. Aggregation of micelles covered with whey proteins starts at a higher pH during acidification, because the iso-electric point of whey proteins is higher than that of caseins.

Recently, Koutina et al. [21-22] studied the effect of the temperature on acid induced gelation of casein micelles in milk and related it to solubilization of the CCP. They found that the critical pH below which the systems gelled increased with increasing temperature: pH 5.2, 5.4 and 5.6 for 40, 50 and 60°C, respectively. The effect of was attributed to changes in the content of calcium and orthophosphate in the micelles with increasing temperature. NMR studies showed that restructuring occurred of the complexes between calcium, orthophosphate and phosphoserines on the caseins in milk during heating [23].

Interestingly, it was found that aggregation and gelation of casein micelles could also be induced by increasing the temperature of milk after decreasing the pH [24-25], but contrary, to the effect of heating milk and subsequently reducing the pH, this process has attracted little attention so far. The critical temperature above which aggregation of micelles occurred in skimmed milk was found to increase with increasing pH from 0°C at pH 4.4 to 30°C at pH 5.2 [24]. Vasbinder, et al. [25] investigated this phenomenon using diffusive wave spectroscopy to detect aggregation of the micelles. They observed that the critical temperature above which aggregation of the micelles occurred increased from about 25°C at pH 5.0 to 50°C at pH 5.4. Comparison between skimmed milk and whey protein free milk did not show major differences indicating that the presence of whey did not influence the onset of gelation of the casein micelles. In addition, it was found that the heating rate did not influence the critical gelation temperature. The authors pointed out that increasing the temperature at fixed pH was not equivalent to decreasing the pH at fixed temperature.

As far as we are aware, thermal gelation of casein micelles above pH 5.6 has not yet been reported before, nor have the mechanical properties and the microstructure of heat-set casein micelle gel been investigated. The objective of the present study was to investigate the phenomenon of heat induced gelation of casein micelles in aqueous solution at fixed pH over a wide range of protein concentration (25-160 $g.L^{-1}$) and pH (5.2-6.7). We have used native

phosphocaseinate powder dispersions in order to obtained dispersion of casein micelles in water free from whey protein, lactose and serum minerals. It was shown that the size and structure of these casein micelles and their behaviour during acidification at room temperature is almost the same as that of caseins micelles in milk [9, 26]. The detailed investigation of heat-induced gelation of pure casein micelles in water presented here can serve as a bench mark for the more complex behaviour of mixtures of casein micelles and whey proteins.

We will show that gels can be formed by casein micelles up to pH 6.5 when the solutions are heated at 90°C. The stiffness of the heat-set gels was studied using oscillatory shear rheology and the microstructure was studied using confocal laser scanning microscopy (CLSM). We will compare the behaviour of casein micelles with that of sodium caseinate (NaCas) that is obtained if the CCP is removed.

2. Materials and Methods.

2.1. Materials. Casein micelles were provided in the form of native phosphocaseinate powder (NPCP) by INRA STLO in Rennes. NPCP was following the method developed by Pierre et al.[27] and Schuck et al.[28]. Briefly, skimmed milk was processed through cross-flow microfiltration to separate the casein micelles from the serum proteins. The micelles were washed with water in diafiltration mode. The solution was then dried in low-temperature conditions through spray-drying. The powder contained 83% (w/w) protein (MAT Kjeldahl), 8.7% (w/w) of ashes including 2.6 % (w/w) of calcium and 1.7% (w/w) of total phosphorus. NPCP has been used as a model for native casein micelles before and after dispersion in water the size and structure of NPCP and its behaviour during acidification is almost the same as that of caseins micelles in milk [9, 26]. The sodium caseinate powder that was used for this study (Lactonat EN, Lactoprot, Kaltenkirchen, Germany) contained 90% (w/w) protein (TNC,

Kjeldahl), 1.3% (w/w) sodium and 0.7% (w/w) phosphorus. The fraction of residual whey protein in either of the casein powders was at most a few wt%.

2.2. Sample preparation. The powder was dispersed in milliQ water containing 3mM of sodium azide (NaN_3) to prevent bacterial proliferation. Fully dispersed suspensions of NPCP were obtained after heating the solution at 50°C during 16h. Alternatively, the micelles were dispersed using a homogenizer at room temperature. No difference in the behaviour of the micelles was observed. About 10% of the protein did not sediment after centrifugation for 2 h at $5x10^4$ g and was therefore not in the form of micelles in the powder. In the concentration range used here less than 5% of micelles that disintegrated over a period of one day after preparation [29]. For NaCas, homogeneous solutions were obtained after heating at 80°C during 30 minutes. The pH was adjusted at 20°C under vigorous stirring by slow addition of aliquots of HCl or NaOH solutions (0.1-1M). The protein concentration was determined by UV-absorbance at 280nm (Varian Cary-50 Bio, Les Ulis, France) using an extinction coefficient of $0.81 L.g^{-1}.cm^{-1}$.

2.3. Confocal Laser Scanning Microscopy (CLSM). CLSM observations were made using a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany) as described in [24].

2.4. Rheology. Oscillatory measurements were performed with two stress-imposed rheometer (TA Instruments Rheolyst, ARG2 and AR2000) using a plate - plate geometry (40 mm, gap 1mm). The temperature was controlled by a Peltier system and the geometry was covered with a mineral oil to avoid water evaporation. Measurements were done at a stress of 0.01 Pa which was within the linear response regime. The frequency was set at 0.1Hz unless otherwise specified. Temperature ramps were done at a rate of 2°C/min except close to the critical gel temperature where they were done at a rate of 0.1°C/min.

3. Results and discussion

3.1. Gelation of casein micelles

3.1.1. Sol-gel state diagram.

A series of aqueous solutions of casein micelles was prepared at room temperature (20°C) at four different concentrations between 25g.L^{-1} and 160g.L^{-1} and at different pH between pH 5.2 and pH 6.7. For pH≤5.2, precipitation was observed at room temperature. The solutions were heated in a water bath during 15 minutes at different temperatures. After cooling to room temperature the samples were observed when tilted and were considered gels if they did not flow. It was found that gelation occurred above a critical temperature (T_c) that increased with increasing pH and decreasing protein concentration, see Figure 1. At C=160 g.L^{-1}, T_c varied little between pH 5.4 and pH 5.8, but increased steeply with increasing pH at higher pH from about 30°C at pH 5.8 to 90°C at pH 6.5. We note that similar results were obtained in trails with a commercial casein micelle powder Promilk 852B, Cremo/Ingrédia (Fribourg, Switzerland). These results show that heat induced gelation of casein micelle solutions occurs at higher pH than was previously realized [24-25].

Reducing the protein concentration to C=100 g.L^{-1} did not have much influence on Tc, but at C=55 g.L^{-1} the critical temperature was significantly higher. Decreasing the protein concentration further to 25g.L^{-1} led to a further increase of T_c, but at this concentration the gels could not support their own weight and the aggregated micelles precipitated in the form of large flocs.

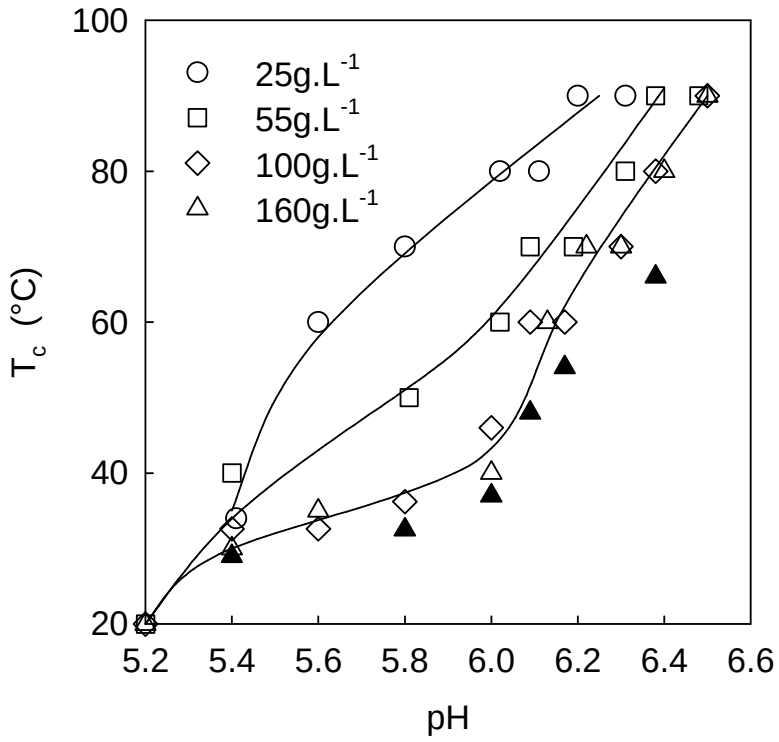

Figure 1. Critical gel temperature of casein micelle suspensions as a function of the pH for different protein concentrations indicated in the figure. Open symbols indicated results obtained by visual observation and filled symbols indicate results obtained from rheology.

Remarkably, the heating time had little influence on T_c. At $T>T_c+5°C$, gels were formed within a minute after the set temperature was reached, whereas at $T<T_c-5°C$ the systems remained liquid even after overnight heating. Only in a very narrow temperature range around T_c slow gelation was observed. The temperature dependence of the gelation rate of casein micelles is much stronger than that of globular proteins. For globular proteins the rate determining step of gelation is denaturation, which is characterized by an activation energy [30]. This means that though the gelation rate increases steeply with increasing temperature, there is no well-defined critical temperature for gelation of globular proteins.

3.1.2. Rheology.

We have studied the rheological behaviour of casein micelle suspensions at C=160 $g.L^{-1}$ as a function of the pH using a slow temperature ramp, see Materials and Methods. Figure 2a shows the temperature dependence of the storage (G') and loss (G") moduli at 0.1 Hz. Unheated, dense suspensions of casein micelles are viscoelastic liquids [31] with G'<G". When the temperature was increased G' increased sharply at a critical temperature and became larger than G". The increase of G' stagnated at higher temperatures except at pH 5.4 where G' decreased after reaching a maximum, which we attribute to syneresis.

The critical temperatures at which G' and G" crossed are compared to T_c obtained by visual observation in Figure 1. The values of T_c obtained by visual observation were a few degrees higher probably because very weak gels do not resist flow when tilted. If G' is plotted as a function of $T-T_c$, see Figure 2b, it can be seen more clearly that the same storage modulus is reached for $T-T_c$ >10°C at all values of the pH except at pH 6.4 for which G' was significantly smaller for reasons that will be discussed below. The frequency dependence of the shear moduli was weak (data not shown) and we will take the value of G' at 0.1 Hz at long heating times as the elastic modulus (G_{el}) of the heat-set gels.

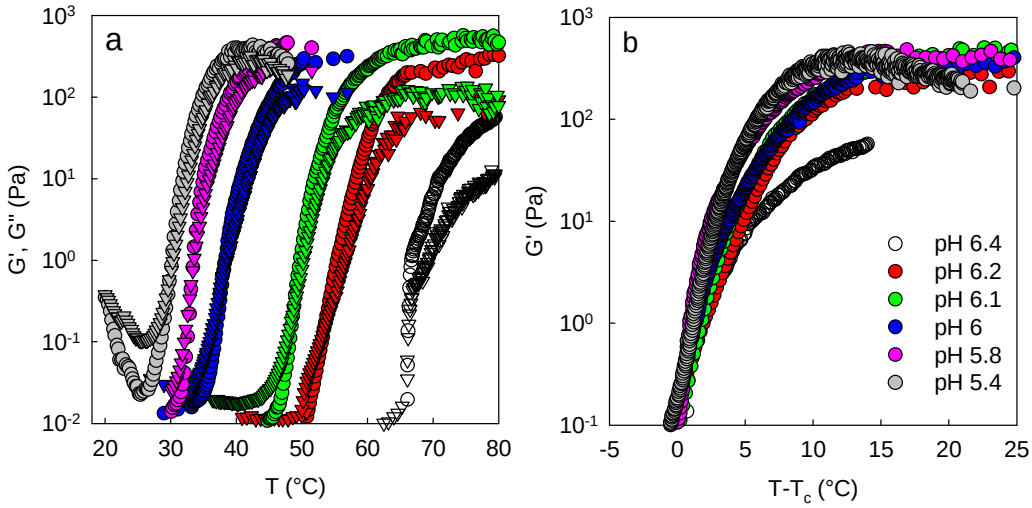

Figure 2a. Storage (circles) and loss (triangles) moduli at 0.1Hz as a function of the temperature during a slow heating ramp for casein micelle suspensions at C=160 g.L^{-1}. For clarity, results obtained far from the critical temperature are not shown.

Figure 2b. Evolution of the storage moduli (G') shown in Figure 2a as a function of the difference between the heating temperature and T_c.

The effect of cooling on G' is shown in Figure 3. At all pH values investigated here, there was little effect of cooling down to 50°C, but further cooling led to a significant increase of the elastic modulus. This temperature dependence suggests that the crosslinks between the micelles involve hydrogen bonds that become stronger with decreasing temperature. The weak temperature dependence between 50°C and 80°C may have been caused by strengthening of hydrophobic interactions that compensated weakening of hydrogen bonds. At 80°C, the gel stiffness did not depend significantly on the pH except at pH 6.4. However, at 20°C, G' was smaller at pH 6.1 and 6.2.

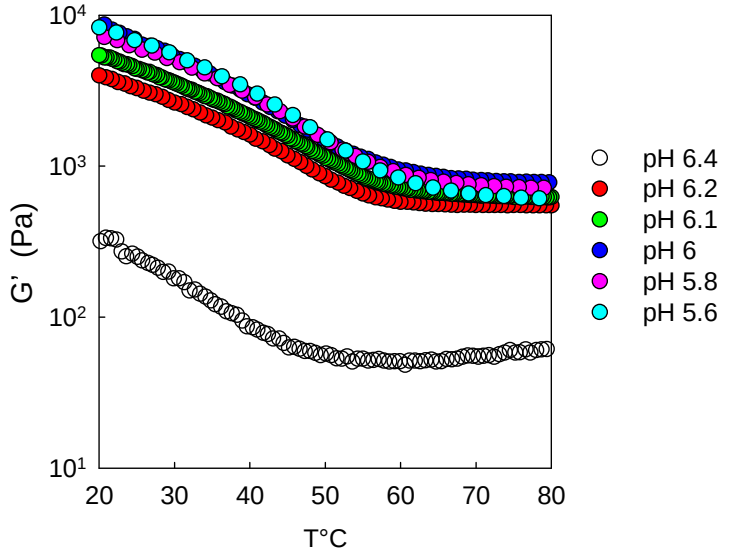

Figure 3. Dependence of the storage (G') on the temperature during cooling for casein micelle gels formed at C=160 g.L^{-1} at different pH at 80°C.

The effect of the heating temperature on gelation close to T_c was studied in more detail at pH 6.0. Figure 4a shows the evolution of G' with time for C=160g.L^{-1} at different temperatures between 35°C and 50°C. For T<35°C, the increase of G' was not significant, whereas at 50°C the gel was formed during the increase of temperature before it had reached 50°C. Between 40°C and 35°C, the rate of gelation decreased, but more importantly G_{el} dropped dramatically, see Figure 4b. It appears that gels with the same stiffness are formed independent of the heating temperature except close to T_c. It is possible that close to T_c not all casein micelles can form bonds and contribute to the network, because there is some dispersity of the size and composition of the micelles. The steep increase of G' would in that case represent the steep increase of the fraction of micelles that can bind to other micelles. The decrease of G_{el} close to T_c may explain the lower stiffness of gels formed at pH 6.4 at 80°C, because 80°C is close to T_c at pH 6.4.

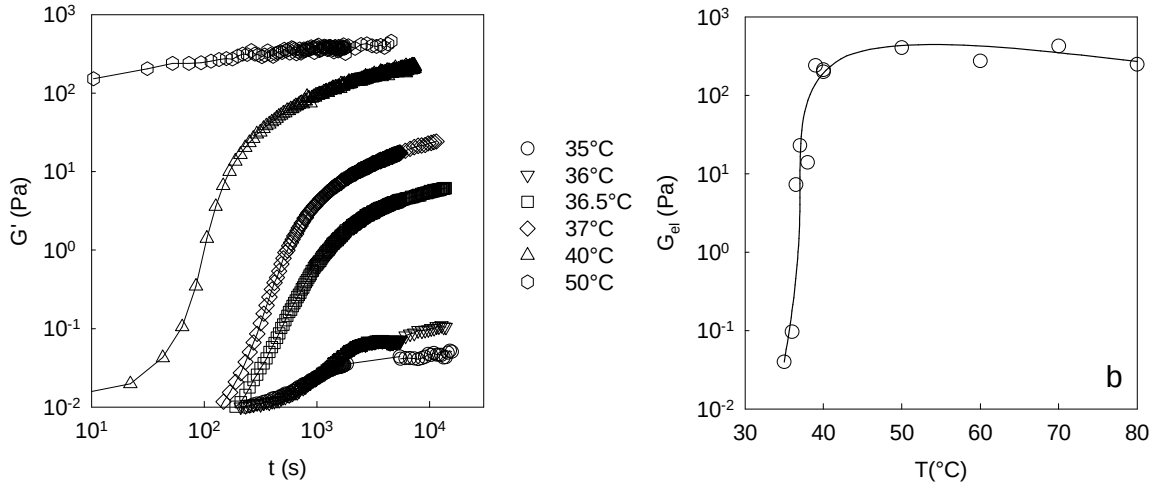

Figure 4a. Storage modulus (G') of casein micelles suspensions at C=160 g.L^{-1} and pH 6.0 as a function of time for different temperatures indicated in the figure.

Figure 4b. Elastic modulus (G_{el}) of gels formed at C=160 g.L^{-1} and pH 6.0 as a function of the heating temperature.

The effect of the casein micelle concentration on the stiffness of the gels was determined at pH 5.6, 6.0 and 6.2 after heating at 80°C, see Figure 5. At this temperature, gelation occurred quickly and G' reached its plateau value after a few minutes. At C<40 g.L^{-1} the gels did not support their own weight and large proteins flocs precipitated. For C≥40 g.L^{-1}, G_{el} increased steeply with increasing protein concentration up to C=60 g.L^{-1} and more weakly at higher concentrations. The initial steep increase is probably caused by reduction of defects and dangling ends of the gel, whereas the weak increase at higher concentrations is caused by incorporating more micelles in the network structure. G_{el} was not influenced by the pH over the range of investigated concentrations, as was already shown for C=160 g.L^{-1} in Figure 2. It is remarkable that as long as the heating temperature is significantly larger than a critical value, gels with the same stiffness are formed even though the net charge density of the caseins and their mineral content are different.

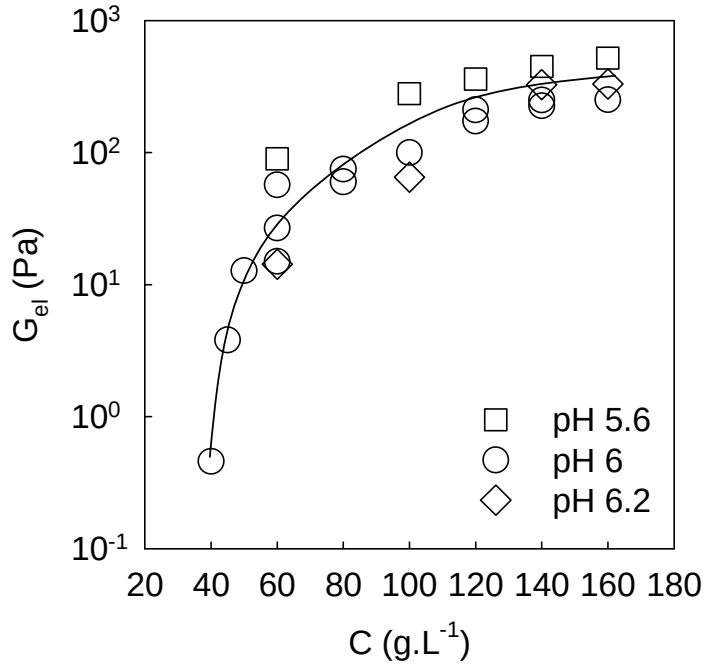

Figure 5. Elastic modulus (G') of heat-set casein micelle gels as a function of the concentration at different pH indicated in the figure.

The concentration dependence of T_c obtained from visual observations and rheology are shown in Figure 6 at three different pH. At all three pH values, T_c varied little for C>100g.L^{-1}, but increased at lower concentrations. Figure 6 also shows that the increase of T_c with increasing pH depends little on the protein concentration. The effect of the pH on T_c is no doubt in part caused by the reduced charge density of the caseins and therefore reduced electrostatic repulsion between the micelles. Reducing the pH also causes more minerals to be released from the micelles [32], which may also influence bond formation. However, NMR measurements showed that down to pH 5.6 less than 10% of the inorganic phosphate is present as free ions for micelle solutions at C=100 g/L [33]. The increase of T_c with decreasing protein concentration may be explained by the fact that the net charge density of proteins is larger for a given pH at lower protein concentrations.

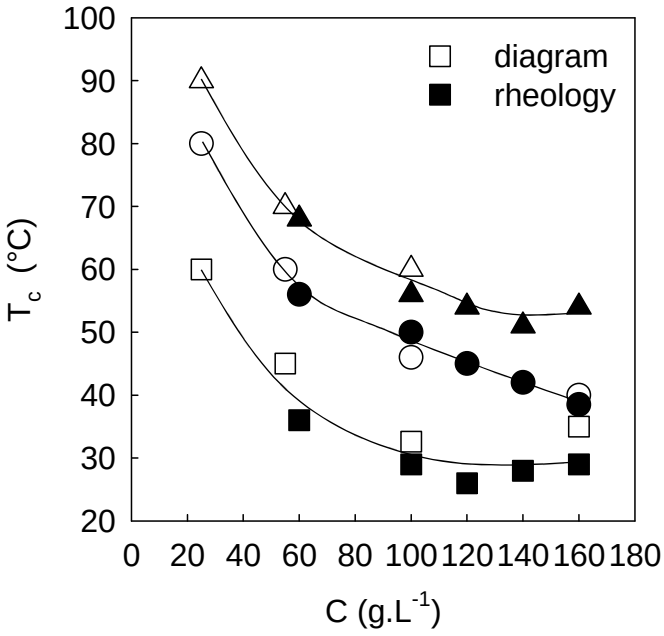

Figure 6. Evolution of the critical temperature (T_c) as a function of the concentration for pH 6.2 (triangles), 6 (circles) and 5.6 (squares).

3.1.3. Microstructure of the gels.

Figure 7 shows CLSM images of casein micelle solutions at C=160 g.L^{-1} and pH 6.0 that were heated for 15 min at different temperatures. At this pH and protein concentration, T_c was determined by rheology to be 35°C, see Figure 4. Below T_c, the suspensions were homogeneous, but for $T>T_c$, they became increasingly heterogeneous with increasing temperature. Similar heterogeneous structures were reported for casein micelle gels formed by acidification at pH 4.6 [18, 34]. The strong dependence of the microstructure on the heating temperature was unexpected, because G' was independent of the heating temperature for T>40°C, see Figure 4b. Stronger attraction between the micelles at higher temperatures may explain the more heterogeneous structure, but this had apparently no influence on the gel stiffness. Similar images were obtained at lower protein concentrations with, as might be expected, denser gel structures at higher protein concentrations.

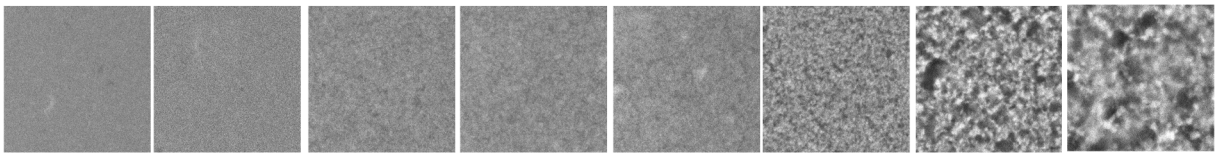

Figure 7. CLSM images (40×40µm) of casein micelles suspensions at 160 g.L^{-1} and pH 6.0 for samples heated at different temperatures indicated under the figure.

3.2. Gelation of sodium caseinate.

In order to gauge the importance of the micellar structure of casein and the presence of calcium phosphate for the gelation process we have investigated the effect of heating on sodium caseinate solutions at different pH. Figure 8 shows the evolution with time of G' for NaCas solutions at C=160 g.L^{-1} and different pH during heating at 80°C. At pH 6.7 an extremely slow increase could be observed, but after overnight heating the system was still a liquid and the viscosity was not significantly higher. However, at pH 6.0 we observed a much stronger increase of G' leading to the formation of weak gels with G'>G". When the pH was further decreased gelation became faster and stiffer gels were formed. This may be due to weaker electrostatic repulsion between the caseins at lower pH, which would allow more bonds to be formed and faster.

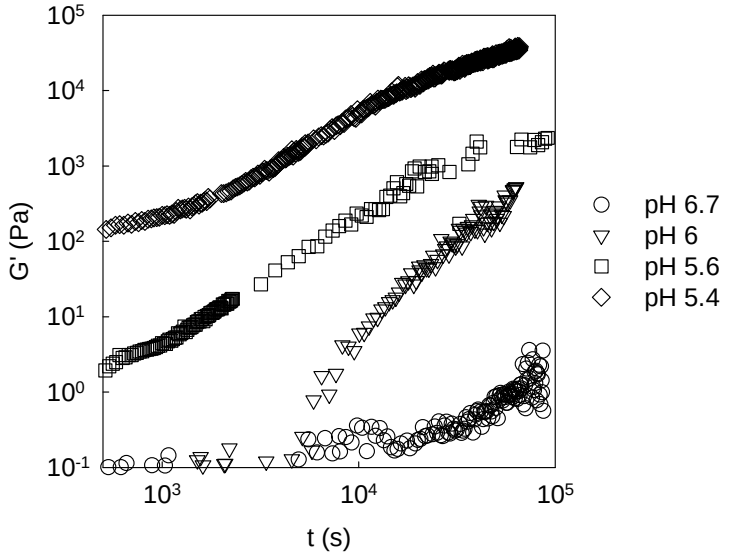

Figure 8. Storage modulus (G') at 0.1 Hz as a function of heating time at 80°C for NaCas suspensions at C=160 g.L^{-1} at different pH indicated in the figure.

The effect of the heating temperature and the protein concentration was investigated at pH 6.0. Figure 9a shows the evolution of G' at three temperatures for C=160 g.L^{-1} and Figure 9b shows the evolution at 80°C for 4 different concentrations. Below 60°C no significant increase of G' was observed after overnight heating, but at higher temperatures gels were formed at a rate that increased with increasing temperature. As was the case for micellar casein, the gels persisted and even became somewhat stiffer when they were cooled. Caseins are rheomorphic proteins and contrary to globular proteins there is no change of their conformation induced by heating. The increase of the gelation rate with increasing temperature can perhaps be related to increased hydrophobic interactions, which are subsequently reinforced by hydrogen bonds. As might be expected, decreasing the protein concentration also led to a decrease of the gelation rate.

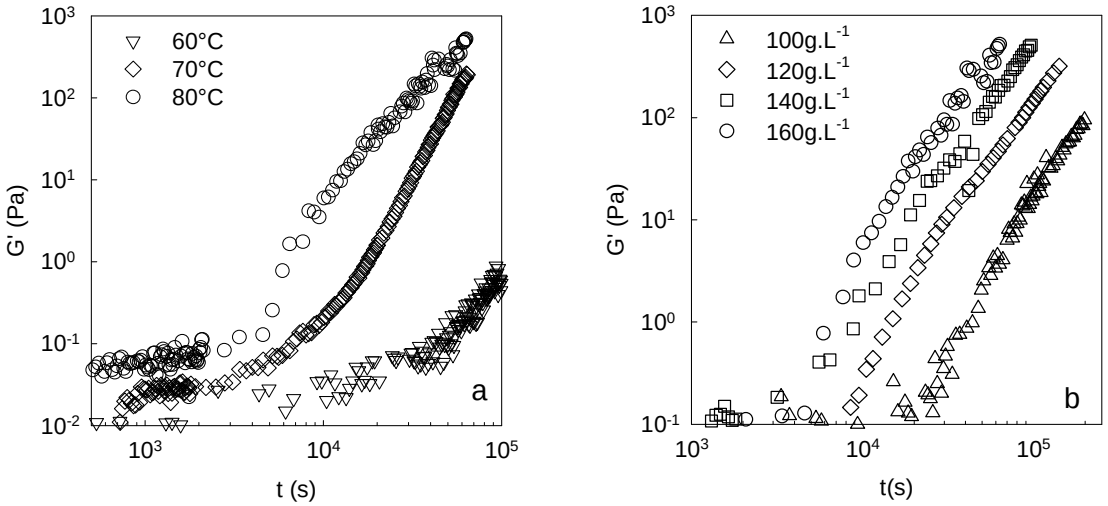

Figure 9a. Storage modulus (G') as a function of time of NaCas suspensions at C=160 g.L^{-1} and pH 6.0 during heating at different temperatures indicated in the figure.

Figure 9b. Storage modulus (G') as a function of time of NaCas suspensions at pH 6.0 and different concentrations indicated in the figure during heating at 80°C.

The gelation process of NaCas in aqueous solution is very different from that of casein micelles. Gelation of NaCas is slow and although there is a strong dependence of the gelation rate on the heating temperature, there is no indication of a critical temperature. Furthermore, the gel stiffness increased with decreasing pH whereas for casein micelles it was independent of the pH. It is clear from the comparison between NaCas and micellar casein gelation that the micellar structure and/or the presence of minerals are very important. In particular the role of calcium and phosphate, of which an increasing fraction is liberated from the micelles when the pH is decreased, may be important. It was found that when calcium phosphate is added to NaCas it forms calcium phosphate nanoclusters involving phosphoserines of the caseins, leading to aggregation of the proteins [35-37]. The aggregation becomes more important with increasing temperature which suggests that hydrophobic interactions play a role. Furthermore, the increase of G' with decreasing temperature suggests that also hydrogen bonds are involved. These three types of interactions also play a role in the stability of the micelles themselves. Anema and Klostermeyer[38] found that for pH 6.5 and pH 6.3 almost no casein was released from the

micelles when milk was heating up to at least 90°C, which would mean that the gelation is not caused by a change in the casein composition of the micelles. However, these measurements were done in the presence of whey proteins which may have affected these findings. In any case, it is very likely that the micellar structure changes when they are heated. We speculate that the subtle balance of interactions in the micelles is perturbed by decreasing the pH and heating, leading to a reorganization of the calcium phosphate complexes with the caseins and perhaps release of caseins. The experimental results show that this reorganization allows bond formation between micelles above a critical temperature.

De Kruif et al.[14, 24] suggested that aggregation of micelles could be described in terms of the adhesive hard sphere model that treats the micelles as spherical colloids with strong short range attractive and repulsive interactions. When the balance between attractive and repulsive interactions reaches a critical value, aggregation occurs. Therefore progressive changes in the balance with increasing temperature will leads to aggregation at a critical temperature. Of course, in reality the micelles are not all identical, which explains why not all micelles aggregate at exactly the same temperature. However, it explains why the sol-gel transition occurs in a narrow range of temperatures. Unfortunately, the present investigation does not enable us to explain the changes of the attractive and repulsive interactions in terms of structural changes of the micelles on a molecular level.

The thermal gelation process will be influenced by the presence of added salt, lactose or whey proteins. As was mentioned in the introduction, heat-induced aggregation of whey proteins and their co-aggregation with caseins will modify the gelation process at temperature above 70°C. Furthermore, we observed in preliminary measurements that the critical gel temperature was higher when 0.1M NaCl was added. The results obtained in this study on pure casen micelles in water can be used as a benchmark to understand thermal gelation in more complex milk systems.

5. Conclusion

Gelation of aqueous solutions of casein micelles can be induced by heating at a fixed pH between pH 5.4 and pH 6.5. However, the gelation process is different from sodium caseinate. Casein micelles at C>100 $g.L^{-1}$ gel within a few minutes above a well-defined critical temperature that is about 30°C between pH 5.4 and pH 5.8 and then increases sharply with increasing pH to 90°C at pH 6.5. The critical temperature increases when the casein concentration is reduced, but self supporting gel are formed down to C=40 $g.L^{-1}$. At lower protein concentrations a sediment of large flocs is formed. The stiffness of the gels increases strongly with increasing protein concentration, but does not depend much on the heating temperature or the pH.

Comparison with heat induced gelation of sodium caseinate solutions shows that the micellar structure and/or the presence of calcium and phosphate is important. Sodium caseinate solutions gel much more slowly than casein micelles at a rate that increases progressively with increasing temperature and decreasing pH. Aggregation of casein micelles is probably caused by restructuring of the micelles induced by changes of the pH and the temperature. Restructuring leads to a progressive change of the balance between repulsive and attractive interaction between the micelles with increasing temperature in favor of the latter and drives aggregation when it reaches a critical value at a critical temperature. Hydrophobic interactions, hydrogen bonding and complex formation between calcium, phosphate and phosphoserines are all involved in the gelation process.

References.

[1] D.G. Dalgleish, On the structural models of bovine casein micelles—review and possible improvements, *Soft Matter* 7 (2011) 2265-2272.

[2] C.G. de Kruif, Caseins, *Progress in Biotechnology* chapter IV 23 (2003) 219-269.

[3] C. Holt, J.A. Carver, H. Ecroyd, D.C. Thorn, Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods, *Journal of Dairy Science* 96 (2013) 6127-6146.

[4] D.S. Horne, Casein micelle structure: models and muddles, *Current Opinion in Colloid & Interface Science* 11 (2006) 148-153.

[5] D.J. McMahon, B.S. Oommen, Casein micelle structure, functions, and interactions, *Advanced Dairy Chemistry*, Springer, New-York, 2013, pp. 185-209.

[6] C.G. de Kruif, E.B. Zhulina, κ-casein as a polyelectrolyte brush on the surface of casein micelles, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 117 (1996) 151-159.

[7] R. Tuinier, C.G. de Kruif, Stability of casein micelles in milk, *Journal of Chemical Physics* 117 (2002) 1290-1295.

[8] D.G. Dalgleish, A.J.R. Law, pH-induced dissociation of bovine casein micelles. II mineral solubilization and its relation to casein release., *Journal of Dairy Research* 56 (1989) 727-735.

[9] M. Famelart, F. Lepesant, F. Gaucheron, Y. Le Graet, P. Schuck, pH-Induced physicochemical modifications of native phosphocaseinate suspensions: Influence of aqueous phase, *Le Lait* 76 (1996) 445-460.

[10] Y. Le Graet, G. Brulé, Les équilibres minéraux du lait: influence du pH et de la force ionique, *Le Lait* 73 (1993) 51-60.

[11] A. Gonzalez-Jordan, P. Thomar, T. Nicolai, J. Dittmer, The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution, *Food Hydrocolloids* 51 (2015) 88-94.

[12] S.G. Anema, E.K. Lowe, Y. Li, Effect of pH on the viscosity of heated reconstituted skim milk, *International Dairy Journal* 14 (2004) 541-548.

[13] D.G. Dalgleish, P.A. Spagnuolo, H.D. Goff, A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy, *International Dairy Journal* 14 (2004) 1025-1031.

[14] C.G. De Kruif, Skim milk acidification, *Journal of Colloid and Interface Science* 185 (1997) 19-25.

[15] S. Marchin, J.-L. Putaux, F. Pignon, J. Léonil, Effects of the environmental factors on the casein micelle structure studied by cryo-transmission electron microscopy and small angle x-ray scattering/ultra small-angle x-ray scattering, *Journal of Chemical Physics* 126 (2007) 126-135.

[16] C. Moitzi, A. Menzel, P. Schurtenberger, A. Stradner, The pH Induced Sol-Gel Transition in Skim Milk Revisited. A Detailed Study Using Time-Resolved Light and X-ray Scattering Experiments, *Langmuir* 27 (2010) 2195-2203.

[17] S. Ahmad, I. Gaucher, F. Rousseau, E. Beaucher, M. Piot, J.F.o. Grongnet, F.d.r. Gaucheron, Effects of acidification on physico-chemical characteristics of buffalo milk: A comparison with cow's milk, *Food Chemistry* 106 (2008) 11-17.

[18] J.A. Lucey, H. Singh, Formation and physical properties of acid milk gels: a review, *Food Research International* 30 (1998) 529-542.

[19] H. Singh, P.F. Fox, Heat stability of milk: pH-dependent dissociation of micellar kappa-casein on heating milk at ultra high temperatures, *Journal of Dairy Research* 52 (1985) 529-538.

[20] L. Donato, F. Guyomarc'h, Formation and properties of the whey protein/$\ kappa $-casein complexes in heated skim milk–A review, *Dairy Science and Technology* 89 (2009) 3-29.

[21] G. Koutina, J.C. Knudsen, U. Andersen, L.H. Skibsted, Temperature effect on calcium and phosphorus equilibria in relation to gel formation during acidification of skim milk, *International Dairy Journal* 36 (2014) 65-73.

[22] G. Koutina, L.H. Skibsted, Calcium and phosphorus equilibria during acidification of skim milk at elevated temperature, *International Dairy Journal* 45 (2015) 1-7.

[23] N.M. Wahlgren, P. Dejmek, T. Drakenberg, A calcium-43 and phosphorus-31 NMR study of the calcium and phosphate equilibria in heated milk solutions, *Journal of Dairy Research* 57 (1990) 355-364.

[24] C.G. De Kruif, S. Roefs, Skim milk acidification at low temperatures: A model for the stability of casein micelles, *Netherlands Milk and Dairy Journal* 50 (1996) 113-120.

[25] A.J. Vasbinder, H.S. Rollema, A. Bot, C.G. De Kruif, Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles, *Journal of Dairy Science* 86 (2003) 1556-1563.

[26] A. Bouchoux, J. Ventureira, G. Gésan-Guiziou, F. Garnier-Lambrouin, P. Qu, C. Pasquier, S. Pézennec, R. Schweins, B. Cabane, Structural heterogeneity of milk casein micelles: a SANS contrast variation study, *Soft Matter* 11 (2014) 389-399.

[27] A. Pierre, J. Fauquant, Y. Le Graet, M. Piot, J.L. Maubois, Préparation de phosphocaséinate natif par microfiltration sur membrane, *Le Lait* 72 (1992) 461-474.

[28] P. Schuck, M. Piot, S. Mejean, Y. Le Graet, J. Fauquant, G. Brulé, J.L. Maubois, Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane, *Le Lait* 74 (1994) 375-388.

[29] P. Thomar, T. Nicolai, Dissociation of native casein micelles induced by sodium caseinate, *Food Hydrocolloids* 49 (2015) 224-231.

[30] T. Nicolai, M. Britten, C. Schmitt, β-Lactoglobulin and WPI aggregates: Formation, structure and applications, *Food Hydrocolloids* 25 (2011) 1945-1962.

[31] A. Bouchoux, B. Debbou, G. Gesan-Guiziou, M.-H. Famelart, J.-L. Doublier, B. Cabane, Rheology and phase behavior of dense casein micelle dispersions, *The Journal of Chemical Physics* 131 (2009) 165106.

[32] Y. Le Graet, F. Gaucheron, pH-induced solubilization of minerals from casein micelles: influence of casein concentration and ionic strength, *Journal of Dairy Research* 66 (1999) 215-224.

[33] A. Gonzalez-Jordan, P. Thomar, T. Nicolai, J. Dittmer, The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution, *Food Hydrocolloids* 51 (2015) 88–94.

[34] J.A. Lucey, C. Dick, H. Singh, P.A. Munro, Dissociation of colloidal calcium phosphate-depleted casein particles as influenced by pH and concentration of calcium and phosphate, *Milchwissenschaft* 52 (1998) 603-606.

[35] E.M. Alvarez, P.H. Risso, C.A. Gatti, M. Burgos, V. Suarez Sala, Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate, *Colloid & Polymer Science* 285 (2007) 507-514.

[36] P. Thomar, D. Durand, L. Benyahia, T. Nicolai, Slow dynamics and structure in jammed milk protein suspensions, *Faraday Discussions* 158 (2012) 325-339.

[37] C.A. Zittle, E.S. Dellamonica, Binding of calcium to casein: influence of pH and calcium and phosphate concentrations, *Archives of Biochemistry and Biophysics* 76 (1958) 342-353.

[38] S.G. Anema, H. Klostermeyer, Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100°C, *J. Agric. Food Chem*. 45 (1997) 1108-1115.

General conclusions and perspectives

The aim of this thesis was to determine the influence of minerals, notably phosphate and calcium on the organization and rheological properties of dense suspensions of sodium caseinate. The investigation was carried out principally using centrifugation, Confocal Laser Scanning Microscopy, rheology and ^{31}P MAS NMR techniques.

When calcium is bound specifically to the proteins, it introduces attractive interactions probably calcium bridging, hydrophobic or hydrogen bonding leading to microphase separation. The microphase separation was marked by the formation of spherical dense protein domains above a critical molar ratio of 6 Ca^{2+} per casein. This phenomenon occurred at lower Ca^{2+} concentrations with increasing the temperature so most likely hydrophobic interactions are involved. Oscillatory shear measurements revealed an increase of the terminal relaxation time and a decrease of the high frequency elastic modulus with increasing Ca^{2+} and protein concentration and with decreasing temperature, which can be explained by the increasing attraction between the caseinate particles. However, when attraction becomes too strong, the formation of large dense protein domains leads to a decrease of the effective volume fraction causing a drop of the terminal relaxation time and the high frequency elastic modulus. The same properties are observed for calcium caseinate suspensions at different concentrations at the same ratio of Ca^{2+} per protein. The interaction is determined by the ratio of Ca^{2+} per protein and not the total amount of calcium ions. The presence of Ca^{2+} induces aggregation of the caseins, but does not lead to gelation by the formation of a system spanning network. Only at high protein concentrations in which the dense and large casein particles are jammed does the system behave as a soft solid.

Addition of monovalent salt up to 250mM does not introduce significant changes of the structure or the viscosity of NaCas suspensions indicating that electrostatic interactions are unimportant. However, above a critical concentration of Na^{+}, attractive interactions due to dehydration of the proteins lead to a strong increase of the viscosity of NaCas and precipitation of NaCas above 3.5M Na^{+}. The same effect was observed using K^{+}. Addition of NaCl mitigates the effect of adding $Ca^{2+,}$ but a large excess of sodium ions is necessary to compete with effectively with calcium ion as the latter binds strongly to the caseins.

The presence of phosphates strongly influences the interaction between caseins when calcium ions are present. When orthophosphate is added to NaCas solutions in the presence of calcium ions, it binds to the casein together with calcium to form complexes. The complexes contain all caseins and possibly have a local structure comparable to that of native casein micelles. However, for the casein micelle, the growth is limited by the κ-casein stabilizing layer. It is possible that calcium phosphate particles comparable to native CCP are formed when orthophosphate and calcium are added to caseinate, but small angle X-ray and neutron scattering experiments need to be done to compare the structure of the complexes with native casein micelles. When orthophosphate is present in excess before $CaCl_2$ is added, it competes with the proteins for binding to calcium and precipitates in the form of large calcium phosphate particles surrounded by an adsorbed protein layer. We did not attempt to determine the type of these large calcium phosphate particles (apatite, brushite, etc.), which necessitates wide angle X-ray scattering experiments. Preliminary experiments, showed that the presence of these large calcium phosphate particles was dependent on the order of incorporation of calcium and phosphate ions in the caseinate solution. The order probably also influences the large scale association of the caseins. Further studies are needed to understand in more detail the effect of the order of adding minerals to caseinate solutions.

When sodium caseinate is mixed with casein micelle, calcium chelation by NaCas leads to dissociation of the micelles. The dissociation was marked by a decrease of turbidity and an increase of the solubility of proteins. However, this state is transient, because the proteins reassemble into large aggregates at a rate that increases with increasing protein concentration. This relatively slow aggregation process is probably induced by the presence of calcium and phosphate that is released from the micelles. A better understanding of this secondary aggregation process in mixtures of NaCas and casein micelles requires further studies.

Casein micelles can also by dissociated by reducing pH below pH 5. When the pH is subsequently increased to pH>6, interaction of the caseins with the calcium and phosphate released from the micelles leads to large scale aggregation. This situation is close to that discussed above in which calcium and orthophosphate are added to caseinate solutions at pH>6. However, the structure of the complexes may depend on the route by which they are produced. Scattering techniques will be needed to study the structure of the complexes on small length scales.

The effect of calcium phosphate was also clear from a comparison between heat-induced gelation of native casein micelles and demineralized sodium caseinate. Casein micelle suspensions gelled rapidly above a critical temperature that increased steeply with increasing pH between pH 5.4 and pH 6.5, whereas caseinate suspensions gelled slowly and only at high temperatures. We speculated that the rapid gelation of casein micelles was induced by calcium and phosphate that was solubilised by reducing the pH and increasing the pH. In this thesis these interactions have been studied in detail only at pH 6.7 and at room temperature. It is clear, however, that the effect calcium phosphate on sodium caseinate strongly depends on the pH and the temperature. Further investigations are needed to elucidate the effects of temperature and the pH on the interaction.

UNIVERSITE BRETAGNE LOIRE

Université du Maine

Thèse de Doctorat

Peggy THOMAR

Structuration des suspensions dense de caséines par les phosphates en présence de calcium, étude de leurs organisations et de leurs propriétés rhéologiques.

Structuration of dense casein suspensions by phosphates in the presence of calcium. A study of their organization and rheological properties.

Résumé

Les caséines, les protéines les plus abondantes du lait, sont associées sous forme de micelle ayant un rayon moyen de 100nm, pontées par du phosphate de calcium colloïdal (CCP). Lorsque le CCP est retiré par précipitation acide, les micelles se désintègrent formant les caséinates de sodium (NaCas), des particules de 10nm.

Les caséines sont très sensibles aux ions calcium et phosphate présents dans certains produits alimentaires qui constituent un levier important dans l'industrie laitière pour contrôler la texture. La viscosité des suspensions de NaCas augmente fortement lorsque la concentration en protéines augmente à cause du remplissage compact par les particules, mais décroît lorsque la température augmente car la fraction volumique efficace des particules diminue. Au-delà d'un ratio critique Ca^{2+}/caséine, les interactions attractives mènent à la formation de micro-domaines denses qui diminuent la solubilité et augmentent la turbidité. Le phénomène est amplifié lorsque la température augmente et modifie les propriétés viscoélastiques des suspensions de NaCas. En large excès, les ions Na^{+} entraînent la dissociation des micro-domaines denses par compétition avec les ions Ca^{2+}. Au-dessus de 0.2M de Na^{+}, la viscosité augmente à cause de la déshydratation des protéines (salting-out). L'orthophosphate (P_i) se lie aux protéines en présence de calcium, ce qui conduit à la formation de complexes. En large excès, le P_i se lie directement au calcium et précipite forme de phosphate de calcium ou sous forme de complexes avec les protéines. Lorsque les micelles des caséines sont acidifiées au-dessus de pH 5,2, le phosphate et le calcium solubilisés interagissent avec les protéines lorsque la température augmente, ce qui conduit à une gélification.

Cette étude a démontré combien la nature du phosphate et du calcium est fondamentale dans l'organisation des caséines en suspension dense.

Mots clés

Caséines, caséinates, calcium, orthophosphate, suspensions denses, rhéologie.

Abstract

In milk, caseins, the main proteins, are associated into a casein micelle (100nm) held together with colloidal calcium phosphate (CCP). When CCP is removed by acid precipitation into sodium caseinate (NaCas), the caseins reassemble into smaller particles, the so-called submicelles (10nm).

Caseins are highly sensitive to calcium and phosphate ions, used in most applications. Challenge for dairy industry is to control the texture, mainly determined by protein interactions. The viscosity of dense NaCas suspensions strongly increased with increasing concentration due to jamming of the particles. Increasing the temperature led to a decrease of the viscosity as the effective volume fraction of the particles decreased. When a ratio of Ca^{2+} is bound by the casein, attractive interactions are introduced and led to the formation of dense micron sized particles that decreased solubility and increased the turbidity. The effect was stronger with increasing temperature. The visco-elastic properties of NaCas suspensions were also modified. The same behaviour was observed for calcium caseinate suspensions if the ratio of Ca^{2+} per casein was equal. Large excess of Na^{+} led to dissociation of the dense protein domains formed by Ca^{2+} by competiton for specific binding sites of caseins. For increasing the Na^{+} concentration above 0.2M the viscosity increased by decreased hydration of the protein. Orthophosphate (P_i) was bound to the caseins with Ca^{2+} leading to the formation of complexes. Large excess of P_i competed with Ca^{2+}. The precipitation of the caseins and large calcium phosphate particles were formed. When casein micelles are acidified above pH 5.2, calcium and phosphate are solubilized and interact with casein leading to the formation of a gel.

This study showed how calcium and phosphate of different origins are fundamental in the organization of caseins in dense suspensions.

Key Words

Casein, caseinate, calcium, orthophosphate, dense suspensions, rheology.